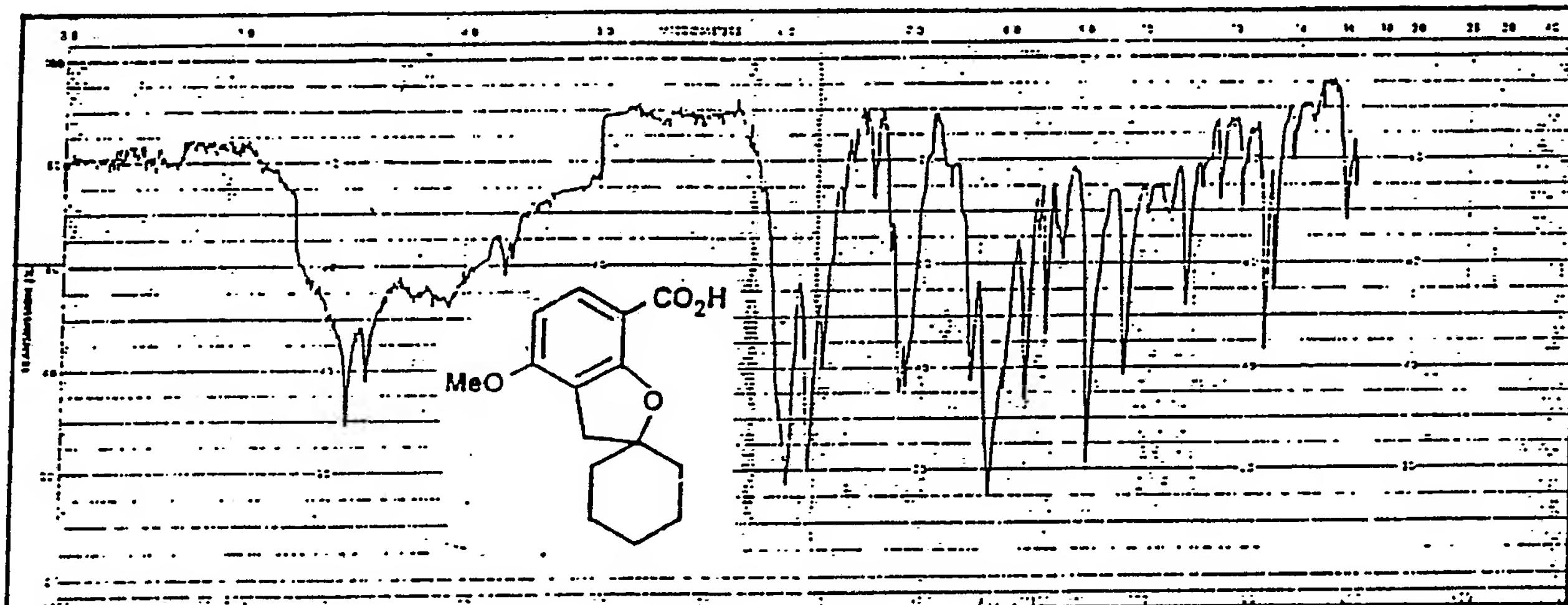




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(60) Parent Application or Grant (63) Related by Continuation US Filed on 623,849 (CIP) 6 December 1990 (06.12.90)		(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).
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(54) Title: COMPOUNDS THAT INHIBIT COMPLEMENT AND/OR SUPPRESS IMMUNE ACTIVITY



(57) Abstract

The present invention is directed to compounds which suppress immune responses and/or selectively inhibit complement. These compounds contain an aromatic ring and are substituted dihydrobenzofurans, spirobenzofuran-2(3H)-cycloalkanes, and their open chain intermediates. The compounds of the present invention, and the pharmaceutically acceptable salts thereof, interrupt the proteolytic processing of C5 to bioactive components, exhibit immunosuppressive activities, and have therapeutic utility in the amelioration of disease and disorders mediated by complement and/or immune activity.

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COMPOUNDS THAT INHIBIT COMPLEMENT
AND/OR SUPPRESS IMMUNE ACTIVITY

1. FIELD OF THE INVENTION

The present invention relates to compounds which
5 inhibit complement and/or possess immunosuppressive activity. In particular, the compounds of the present invention, and the pharmaceutically acceptable salts thereof, selectively inhibit complement at the C5 step of complement activation. The compounds of the invention are
10 substituted dihydrobenzofurans, spirobenzofuran-2(3H)-cycloalkanes, and their open chain intermediates that exhibit such inhibitory activities. Compounds of the invention also include 6,7-disubstituted spirobenzofuran-
15 2(3H)-cycloalkanes and 4-substituted spirobenzofuran-2(3H)-cycloalkanes. The invention relates to the use of these compounds for therapy of immune and/or inflammatory disorders.

2. BACKGROUND OF THE INVENTION

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2.1. THE COMPLEMENT SYSTEM

The complement system is a group of proteins that constitutes about 10 percent of the globulins in the normal serum of humans (Hood, L.E. et al. 1984, Immunology, 2d Edition, The Benjamin/Cummings Publishing Co., Menlo Park, California, p. 339). Complement (C) plays an important role in the mediation of immune and allergic reactions (Rapp, H.J. and Borsos, T., 1970, Molecular Basis of Complement Action, Appleton-Century-Crofts 25 (Meredith), New York). The activation of C components leads to the generation of a group of factors, including chemotactic peptides that mediate the inflammation associated with complement-dependent diseases. The sequential activation of the complement cascade may occur via the 30 classical pathway involving antigen-antibody complexes, or 35

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by an alternative pathway which involves the recognition of certain cell wall polysaccharides. The activities mediated by activated complement proteins include lysis of target cells, chemotaxis, opsonization, stimulation of vascular and other smooth muscle cells, degranulation of mast cells, increased permeability of small blood vessels, directed migration of leukocytes, and activation of B lymphocytes, macrophages and neutrophils (Eisen, H.N., 1974, Immunology, Harper & Row, Publishers, Inc., 10 Hagerstown, Maryland, p. 512).

During proteolytic cascade steps, biologically active peptide fragments, the anaphylatoxins C3a, C4a, and C5a (See WHO Scientific Group, WHO Tech. Rep. Ser. 1977, 606, 5 and references cited therein), are released from the third (C3), fourth (C4), and fifth (C5) native complement components (Hugli, T.E. CRC Crit. Rev. Immunol. 1981, 1, 321; Bult, H. and Herman, A.G. Agents Actions 1983, 13, 405). The C5a fragment, a cationic peptide derived from the first 74 amino acids of the amino-terminus of the C5 alpha subunit (Tack, B.F. et al. Biochemistry 1979, 18, 1490), is of particular pathological relevance. Regulation of C5a activity is by the endogenous plasma enzyme carboxypeptidase N (E.C. 3.4.12.7), which rapidly removes the carboxy-terminal arginine from C5a, producing the less potent but still active C5a des Arg. Reported effects of C3a and C5a upon specific immune responses are listed in Table I.

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TABLE I

**EFFECTS OF COMPLEMENT COMPONENTS
C3a AND C5a ON SPECIFIC IMMUNE RESPONSES**

5

<u>Immune Response</u>	<u>C3a</u>	<u>C5a/C5a des Arg</u>
Specific antibody production in response to sheep red blood cells	Suppression	Enhancement
10 Polyclonal antibody production in response to Fc antibody fragment	Suppression	Enhancement
T cell proliferation in response to tetanous toxoid	Suppression	Enhancement
15 T cell proliferation in mixed lymphocyte reaction	No effect	Enhancement
T cell-mediated cytotoxicity	Suppression	Enhancement
20 Among the wide variety of biological activities exhibited by C5a are contraction of smooth muscle (Wissler, J.H. Eur. J. Immunol. 1972, 2, 73), degranulation of mast cells (Johnson, A.R. et al., Immunol. 1975, 28, 1067), secretion of azurophilic granular enzymes from polymorphonuclear 25 neutrophils (PMN) (Webster, R.O. et al., Immunopharmacol. 1980, 2, 201), and the chemotaxis of PMN (Wissler, J.H. Eur. J. Immunol. 1972, 2, 73; Becker, E.L. Trends Pharmacol. Sci. 1983, 4, 223) (Table II).		

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TABLE II

BIOLOGICAL EFFECTS OF C5a

- 5 I. Stimulation of neutrophil functions
 involved in inflammation
 A. chemotaxis
 B. chemokinesis
10 C. aggregation
 D. lysosomal enzyme release
 E. generation of toxic oxygen products
II. Smooth muscle effects
 A. stomach smooth muscle contraction
15 B. vasodilation
III. Promotion of histamine release
 A. mast cells
 B. basophils
IV. Immunoregulatory effects
-

20 The active chemotactic factor in vivo is considered to be
 C5a des Arg (Becker, E.L. Trends Pharmacol. Sci. 1983, 4,
 223).

25 The C5a or C5a des Arg fragments have been implicated
 in the infiltration of PMN (the chemotactic effect) in
 rheumatoid arthritis, certain forms of glomerulonephritis,
 experimental vasculitides such as the Arthus reaction, the
 acute pneumonitis produced by the instillation of chemo-
 tactic factors into the lungs of experimental animals with
30 resulting release of leukotrienes C-4 and D-4 (LTC₄ and
 LTD₄), etc. In addition, the interactions between C5a and
 neutrophils have been considered to underlie tissue damage
 in several clinical situations. For instance, there
35 exists a growing body of evidence for the role of

oxygen-derived free radicals in mediating myocardial tissue injury during myocardial ischemia and, in particular, during the phase of myocardial reoxygenation and reperfusion. Among a number of possible sources of these 5 radicals, the polymorphonuclear neutrophil has been the focus of primary attention. Studies have documented that neutrophil depletion or suppression of neutrophil function results in a significant salvage of myocardial tissue that is subjected to a period of regional ischemia followed by 10 reperfusion (Simpson, P.J. and Lucchesi, B.R. J. Lab. Clin. Med. 1987, 110(1), 13-30). Neutrophil depletion in dogs resulted in significantly smaller myocardial infarcts after 90 minute occlusion with 24 hour reperfusion (Jolly, S.R. et al. Am. Heart J. 1986, 112, 682-690).

15 One study documented the activation of complement and generation of oxygen-derived free radicals during cardio-pulmonary bypass. The administration of protamine during cardiopulmonary bypass further activated complement (Cavarocchi, N.C. et al., Circulation 1986, 74, 130-133; 20 Kirklen, J.K. et al. J. Thorac. Cardiovasc. Surg. 1983, 86, 845-857). Also, recombinant tissue plasminogen activator (r-TPA), which in recent clinical trials has been found to be an effective thrombolytic agent in patients with acute myocardial infarction, was shown to activate 25 complement. A striking increase in the level of C4a, C3a, and C5a was found in patients receiving r-TPA as compared to the level of these complement peptides before administration of the drug (Bennett, W.R. et al. J. Am. Coll. Cardiol. 1987, 10(3), 627-632). Schafer and co-workers 30 were able to positively identify the deposition of terminal C5b-9 complement complex in myocardial cells located within zones of infarction in human tissue (J. Immunol. 1986, 137(6), 1945-1949). Likewise, the selective accumulation of the first component of complement and leukocytes 35 in ischemic canine heart muscle has been found (Rosen,

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R.D. et al. Circ. Research, 1985, 57, 119-230). In one study, the depletion of complement was found to increase the blood flow in ischemic canine myocardium. This increased blood flow was found, in turn, to increase the supply and utilization of oxygen in complement depleted animals versus control animals (Grover, G.J. and Weiss, H.R. Basic Res. Cardio. 1987, 82(1), 57-65). Complement activation is also believed to initiate adult respiratory distress syndrome (ARDS). This syndrome, also known as adult respiratory failure, shock lung, diffuse alveolar damage, or traumatic wet lungs, is characterized clinically by the rapid onset of severe life-threatening respiratory insufficiency that is refractory to oxygen therapy. (Miescher, P.A. and Muller-Eberhard, H.J., eds., 1976, Text Book of Immunopathology, 2d Ed., Vols. I and II, Grune and Stratton, New York; Sandberg, A.L., 1981, in Cellular Functions in Immunity and Inflammation, Oppenheim, J.J. et al., eds. Elsevier/North Holland, New York, p. 373; Conrow, R.B. et al. J. Med. Chem. 1980, 23, 242; Regal, J.F.; and Pickering, R.H. Int. J. Immunopharmacol. 1983, 104, 617). Some of the clinical implications of C5a release are listed in Table III.

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30

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TABLE III
CLINICAL IMPLICATIONS OF C5a RELEASE

- Rheumatoid Arthritis
- Acute Gouty Arthritis
- 5 Acute Immunological Arthritis
- Pulmonary Disorders
- Adult Respiratory Distress Syndrome
 - Pulmonary Dysfunction - Hemodialysis
 - Chronic Progressive Pulmonary Disease-Cystic Fibrosis
- 10 Byssinosis
- Asbestos-Induced Inflammation
 - Inflammation of Systemic Lupus Erythematosus
 - Inflammation of Glomerulonephritis
 - Purtscher's Retinopathy
- 15 Hemorrhagic Pancreatitis
- Renal Cortical Necrosis
 - Primary Biliary Cirrhosis Inflammation
 - Nephropathology
 - Cranial Nerve Damage in Meningitis
- 20 Tumor Cell Metastasis
- Extended Tissue Destruction in Myocardial Infarction
 - Extended Tissue Destruction in Burns
-

25 2.2. CELL-MEDIATED IMMUNE RESPONSES

A variety of immune responses independent of the complement system are known to be mediated by specifically reactive lymphocytes. These responses may give rise to autoimmune diseases, hypersensitivity, or simply allergic reactions. Some examples of these responses include delayed-type hypersensitivity, allograft rejection, graft versus host disease, drug allergies, or resistance to infection. Autoimmune disorders may include atrophic gastritis, thyroiditis, allergic encephalomyelitis, 35 gastric mucosa, thyrotoxicosis, autoimmune hemolytic

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anemia, and sympathetic ophthalmia (Eisen, H.N., 1979, Immunology, Harper and Row, Hagerstown, Maryland, pp. 557-595).

5 2.3. ORGANIC COMPOUNDS WHICH INHIBIT COMPLEMENT,
 AMELIORATE INFLAMMATION, AND/OR POSSESS
IMMUNOSUPPRESSIVE ACTIVITY

Many chemicals have been reported to diminish complement-mediated activity. Such compounds include: amino acids (Takada, Y. et al. Immunology 1978, 34, 509); phosphonate esters (Becker, L. Biochem. Biophys. Acta 1967, 147, 289); polyanionic substances (Conrow, R.B. et al. J. Med. Chem. 1980, 23, 242); sulfonyl fluorides (Hansch, C.; Yoshimoto, M. J. Med. Chem. 1974, 17, 1160, and references cited therein); polynucleotides (DeClercq, P.F. et al. Biochem. Biophys. Res. Commun. 1975, 67, 255); pimaric acids (Glovsky, M.M. et al. J. Immunol. 1969, 102, 1); porphines (Lapidus, M. and Tomasco, J. Immunopharmacol. 1981, 3, 137); several antiinflammatories (Burge, J.J. et al. J. Immunol. 1978, 120, 1625); phenols (Muller-Eberhard, H.J. 1978, in Molecular Basis of Biological Degradative Processes, Berlin, R.D. et al., eds. Academic Press, New York, p. 65); and benzamidines (Vogt, W. et al. Immunology 1979, 36, 138). Some of these agents express their activity by general inhibition of proteases and esterases. Others are not specific to any particular intermediate step in the complement pathway, but, rather, inhibit more than one step of complement activation. Examples of the latter compounds include the benzamidines, which block C1, C4 and C5 utilization (Vogt, W. et al. Immunol. 1979, 36, 138).

2.4. SPIROBENZOFURAN-2(3H)-CYCLOALKANES AND K-76

K-76 is a fungal metabolite from Stachybotrys complementi nov. sp. K-76. Metabolite K-76 has a drimane

skeleton combined with a benzene ring attached through a spirofuran, and has been determined as 6,7-diformyl-3',4',4a',5',6',7',8',8a'-octahydro-4,6',7'-trihydroxy-2',5',5',8a'-tetramethyl spiro [1' (2'H) -naphthalene-2(3H) -5benzofuran] (Kaise, H. et al. J. Chem. Soc. Chem. Commun. 1979, 726). The monocarboxylic acid derivative, K-76 COOH, is obtained when K-76 is selectively oxidized by silver oxide (Corey, E.J. and Das, J. J. Amer. Chem. Soc. 1982, 104, 5551).

10 Both K-76 and K-76 COOH have been shown to inhibit complement mainly at the C5 step (Hong, K. et al. J. Immunol. 1979, 122, 2418; Miyazaki, W. et al. Microbiol. Immunol. 1980, 24, 1091). In a classical hemolytic reaction system, hemolysis of sensitized sheep erythrocytes 15 by guinea pig serum was reduced 50% by K-76 at 7.45×10^{-5} M, or K-76 COONa at 3.41×10^{-4} M (Hong, K. et al. J. Immunol. 1979, 122, 2418; Miyazaki, W. et al. Microbiol. Immunol. 1980, 24, 1091). Similar results were observed 20 in a hemolytic reaction system via the alternative pathway of complement activation.

Both K-76 and K-76 COOH prevented the generation of a chemotactic factor from normal human complement (Bumpers, H. and Baum, J. J. Lab. Clin. Med. 1983, 102, 421). K-76 has been shown to reduce the amount of protein excreted in 25 urine of rats with nephrotoxic glomerulonephritis (Iida, H., et al., Clin. Exp. Immunol. 1987, 67, 130-134), and is reported to greatly increase the survival of mice with a spontaneous systemic lupus erythematosis-like disease and to suppress Forssman shock in guinea pigs and mice 30 (Miyazaki, W. et al. Microbiol. Immunol. 1980, 24, 1091). At high concentrations of K-76 or K-76 COOH, some inhibition of the reactions of C2, C3, C6, C7, and C9 with their respective preceding intermediaries is exhibited. However, both compounds' inhibitory action is mainly the generation 35 in vitro of EACl, 4b,2a,3b,5b (sensitized sheep

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erythrocytes carrying the indicated complement components) from C5 and EAC_{1,4b,2a,3b}; the acceleration of the decay of any EAC_{1,4b,2a,3b,5b} present; and blocking generation of the chemotactic peptides (Hong, K. et al. J. Immunol. 5 1981, 127, 109; Ramm, L.E., et al. Mol. Immunol. 1983, 20, 155).

K-76 COOH is also reported to be an anti-hepatitic agent (West German Patent Application, Publication No. 3,031,788, published March 12, 1981, by Shinohara, M. et 10 al.), and possesses the ability to inhibit antibody-dependent cell-mediated cytotoxicity and natural killer lytic activity (Hudig, D. et al. J. Immunol. 1984, 133, 408-413). K-76 or K-76 COOH has also been reported to inhibit the C3b inactivator system of complement (Hong, K. 15 et al. J. Immunol. 1981, 127, 104-108). Semi-synthetic derivatives of K-76 have been patented as anti-allergy, anti-tumor, and anti-nephritic agents (Belgium Patent No. 867,095, published November 16, 1978, by Shinohara, M. et al.). The isolation of K-76, its uses in the treatment of 20 autoimmune diseases, and the preparation of its derivatives have been described in a number of patents (See Japanese Patent Applications (Kokai), Publication Nos. 54 092680 (published July 23, 1979), 54 106458 (published August 21, 1979), 57 083281 (published May 25, 1982), by 25 Shinohara, M. et al.; Japanese Patent (Kokoku) No. 85 030289 (published March 20, 1979)).

A number of additional compounds which contain the substructure of a spirobenzofuran-2(3H)-cycloalkane are known. These compounds include griseofulvin (Weinberg, 30 E.D., 1981, in Principles of Medicinal Chemistry, 2d Ed., Foye, W.O., ed., Lea & Febiger, Philadelphia, PA., p. 813), isopannarin (Djura, P. and Sargent, M.V. Aust. J. Chem. 1983, 36, 1057), and metabolites of Siphonodictyon coralli-phagum (Sullivan, B., et al. Tetrahedron 1981, 37, 35 979).

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The general synthetic methodology utilized for the synthesis of the compounds of the present invention involves regioselective aromatic lithiation reactions. Numerous oxygen-containing heterocycles have been made using regioselective aromatic lithiation reactions, but few preparations of dihydrobenzo[b]furans have been described (Narasimhan, N.S. and Mali, R.S. *Synthesis* 1983, 957). Three reported syntheses of K-76 itself (Corey, E.J. and Das, J.J. *J. Am. Chem. Soc.* 1982, 104, 5551; McMurray, J.E. et al. *Am. Chem. Soc.* 1985, 107, 2712 Mori, K. et al. *Ann. Chem.* 1988, 107-119) utilize metalation techniques in the coupling of the terpenoid portion to the aromatic moiety, but neither provide sufficient flexibility to allow for analog preparations via subsequent elaboration of the aromatic ring.

3. SUMMARY OF THE INVENTION

The present invention is directed to compounds which suppress immune responses and/or selectively inhibit complement. In a specific embodiment, such compounds interrupt the proteolytic processing of C5 to bioactive components, blocking the release of C5a. The compounds of the present invention also exhibit immunosuppressive activities, such as for example, the ability to inhibit natural killer activity, lymphocyte proliferations, and T cell activation. The compounds of the present invention have therapeutic utility in the amelioration of disease and disorders mediated by complement and/or immune activity. In specific embodiments, they may be used for the treatment of autoimmune disease or the many diseases associated with the "inappropriate" activation of the complement system. In specific embodiments, a compound of the invention has greater activity than the natural product K76.

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The invention further provides improved synthetic routes for the preparation of the compounds.

In one embodiment of the present invention, compounds are provided which have selectivity in inhibition of C5a release. In particular, such compounds can have utility in limiting the extent of trauma-induced tissue destruction, in the prevention and/or treatment of adult respiratory distress syndrome and damage induced by ischemic heart conditions.

10 In other specific embodiments, compounds of the invention which exhibit immunosuppressive activity can be used in the prevention and/or treatment of autoimmune disease or the rejection of transplanted organs and/or tissues.

15 A further embodiment of this invention includes the combined therapy that can be obtained by treating patients with disorders that are routinely treated with thrombolytic agents such as tissue plasminogen activator, streptokinase or urokinase (e.g. myocardial infarction patients) 20 with a combination of the compounds of this invention and the routinely administered thrombolytic compounds.

The present invention is also directed to pharmaceutical compositions comprising such compounds or the salts thereof.

25

3.1 DEFINITIONS

As used herein, the following abbreviations and terms shall have the meanings indicated:

	<u>n</u> -BuLi	= <u>n</u> -butyllithium
30	<u>t</u> -BuLi	= <u>tert</u> -butyllithium
	<u>t</u> -BuSLi	= lithium <u>tert</u> -butylthiolate
	C	= complement
	CHO	= Chinese hamster ovary
	CPM	= counts per minute
35	Et ₂ O	= diethyl ether

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	HMPA	= hexamethylphosphoric triamide
	IgG	= immunoglobulin G
	ⁱ PrOH	= isopropanol
	IR	= infrared
5	K-76 COOH	= the monocarboxylic acid derivative of K-76
	K-76 COONa	= the sodium salt of the monocarboxylic acid derivative of K-76
10	LAH	= lithium aluminum hydride
	MOM	= methoxymethyl group
	NK	= natural killer
	NMR	= nuclear magnetic resonance
	PBL	= peripheral blood lymphocyte(s)
15	PCC	= pyridinium chlorochromate
	PHA	= phytohemagglutinin
	PMN	= polymorphonuclear cells
	RLi	= alkyllithium
20	THF	= tetrahydrofuran
	TLC	= thin layer chromatography
	TMEDA	= N,N,N',N'-tetramethyl-ethylenediamine
	TMS	= tetramethylsilane
25	TriMEDA	= N,N,N' trimethylethylenediamine

The term "bioisosteric" group, as used in the present invention, describes an alternative chemical group whose electronic configuration is substantially analogous with the group to be replaced such that the polarity and charge of the whole molecule do not change. However, variations in the size, number of atoms or electron structure of the bioisosteric group (or "bioisostere") are permitted which variations may affect its function. Bioisosteres may be

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acidic (e.g., capable of releasing a proton and, subsequently, bearing a negative charge), basic (e.g., capable of being protonated and, subsequently, bearing a positive charge) or neutral (e.g., not normally capable of functioning as an acidic or basic group).

Unless otherwise stated or indicated, the term "alkyl" as used herein refers to methyl, ethyl, and *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, or *tert*-butyl groups. The term "alkanol" denotes a compound derived from coupling an alkyl group and hydroxyl radical. Similarly, the term "alkoxy" refers to methoxy, ethoxy, *n*-propyloxy, isopropyloxy, *n*-, iso-, *sec*, and *tert*-butoxy, phenoxy, benzyloxy groups and substituted derivatives thereof. The term "lower" refers to the numerical range of 1 to 4 carbon atoms and includes linear or branched skeletons.

Unless otherwise stated or indicated, the term "halogen" as used herein includes fluorine, chlorine, bromine, and iodine.

Unless otherwise stated or indicated, a given structure, formula, or nomenclature for the substituted dihydrobenzofuran analogs of this invention shall subsume all stereoisomers thereof.

Unless otherwise stated or indicated, a reference made to a final compound of the invention which is a carboxylic acid, is also meant to include the salt form of such carboxylic acid such as alkali and alkaline-earth metal salts obtained therefrom.

30

4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates the infrared spectrum of compound 11a. The spectrum is presented for the compound pelletized in potassium bromide.

FIGURE 2 demonstrates the inhibition of complement peptide C5a or C3a production by compound 11a. The inhibi-

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bition (as described in Section 6.6, infra) of C5a and C3a production is shown as a function of compound 11a concentration.

FIGURE 3 demonstrates the inhibition of complement-mediated hemolysis by compound 11a. Inhibition of complement-mediated hemolysis, assayed as described in Section 6.6.2 infra, is shown as a function of compound 11a concentration.

FIGURE 4 demonstrates the inhibition of proliferation of peripheral blood lymphocytes (PBL) by compound 11a. ³H-Thymidine incorporation by PHA-stimulated PBL is shown as a function of compound 11a concentration.

FIGURE 5 demonstrates the inhibition of proliferation of PBL by compound 11a. ³H-Thymidine incorporation by antiCD3 antibody-stimulated PBL is shown as a function of compound 11a concentration.

FIGURE 6 demonstrates the inhibition of interleukin-2 receptor (IL-2R) release from PBL by compound 11a. The level of IL-2R in the supernatant of PBL cultures stimulated with anti-CD3 antibody, in the presence of compound 11a, was measured by use of an enzyme-linked immunosorbent assay, and is shown as a function of compound 11a concentration.

FIGURE 7 shows the inhibition of CD8 protein release from PBL by compound 11a. The level of CD8 in the supernatant of PBL cultures stimulated with anti-CD8 antibody, in the presence of compound 11a, was measured by use of an enzyme-linked immunosorbent assay, and is shown as a function of compound 11a concentration.

FIGURE 8 demonstrates the inhibition of complement-mediated hemolysis by the disubstituted spirobenzofuran compounds 62, 66, and 68. Inhibition is shown as a function of compound concentration .

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5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compounds which inhibit complement and/or possess immunosuppressive activity. The compounds of the invention contain an aromatic 5 ring and are substituted dihydrobenzofurans, spirobenzonfuran-2(3H)-cycloalkanes, and their open chain intermediates. In particular, such compounds can be partial analogs of the fungal metabolite K-76.

The complement inhibitors of the invention inhibit C5 activation, that is, the proteolytic generation of bioactive complement fragments C5a and C5b from C5. Such compounds have value in the treatment or prevention of diseases or disorders associated with undesirable or inappropriate activation of the complement system. In specific embodiments, the compounds of the invention can be used in the treatment of inflammatory disorders. They may also be used for the treatment of cardiovascular disease.

The present invention also relates to compounds which 20 possess immunosuppressive activity. In particular, such compounds inhibit immune responses. In specific embodiments, the compounds of the invention can inhibit the killing activity of mononuclear cells, lymphocyte proliferation and/or activation. The immunosuppressive compounds of the invention can be valuable in the treatment 25 of various immune disorders.

Furthermore, the compounds of the invention may possess one or more of the K-76-like activities described supra in Section 2.4 and in the references cited therein.

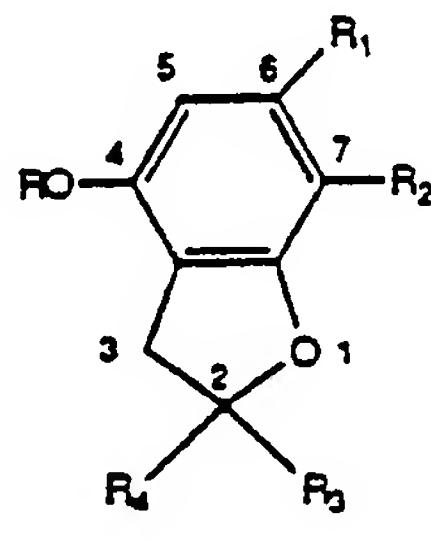
30 The compounds of the present invention, and the intermediates and methods used in their preparation, are described in detail below.

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**5.1. COMPOUNDS WHICH INHIBIT COMPLEMENT
AND/OR SUPPRESS IMMUNE ACTIVITY**

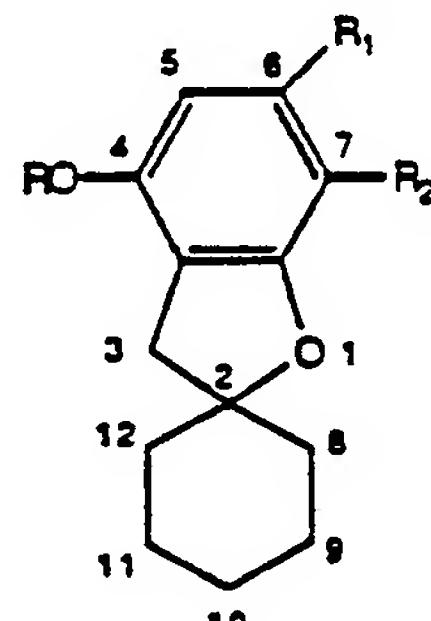
The present invention relates to organic compounds which inhibit complement and/or suppress immune activity. The organic compounds of the invention comprise substituted dihydrobenzofurans of the general formula 3 and substituted spirobenzofuran-2(3H)-cycloalkanes of the general formula 4. The groups represented by R and R₁-R₄ include, among others, hydrogen and linear or branched lower alkyl groups having 1 to 4 carbon atoms as defined previously in Section 3.1, supra. In addition, R₁-R₄ may each

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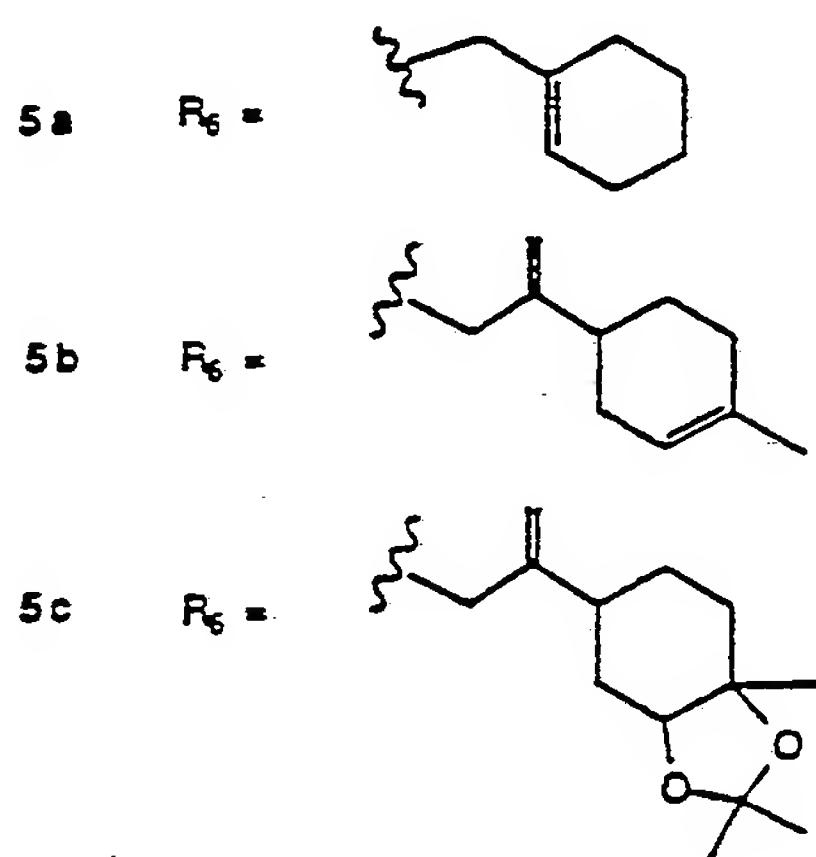
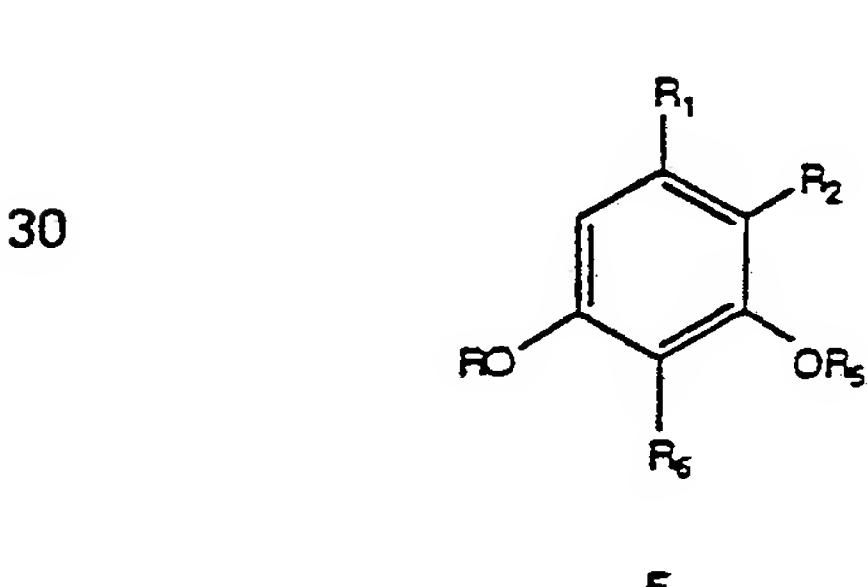
independently represent halogen, amino, amidic, hydroxyl, hydroxyalkyl, alkyloxy, nitro, formyl, acetal, carboxylic acid, trifluoroacetyl, N-substituted lower alkyl carbamoyl, substituted vinyl having up to 10 carbon atoms, an alkylidene group having up to 20 carbon atoms, an aliphatic acyl, a substituted aliphatic acyl, an aromatic acyl, a substituted aromatic acyl, a sulfamoyl, an aminomethyl, N-(lower alkyl)aminomethyl, a N, N-di(lower alkyl) aminomethyl, a heterocyclic ring bearing at least one heteroatom selected from nitrogen, oxygen or sulfur (e.g., a tetrazole or oxazoline group), an N-acylcarbamoyl, an amidino or a hydrazide. Moreover, R₁, R₂ and the carbon atoms to which they are attached may together form a five- or six-membered ring (e.g., a cyclic

-18-

anhydride, such as a phthalic anhydride derivative; a lactone; or a hydroxy-substituted lactone).

Other groups which may be represented independently by R₃ and R₄ include hydrocarbons of 4 to 24 carbon atoms which may be of medium-length, long-chain, linear, branched, cyclic, saturated, unsaturated, unsubstituted, or heteroatom substituted. Moreover R₃, R₄, and the carbon atom to which they are attached may form a cyclic hydrocarbon group of 5-24 carbon atoms which may include a five-, six-, or seven-membered saturated or unsaturated ring comprised exclusively of carbon and hydrogen, or in combination with a heteroatom. The ring may be unsubstituted or may contain extra-cyclic heteroatom or hydrocarbon substituents.

This invention also relates to synthetic open chain intermediate compounds of the general formula 5 wherein R, R₁, and R₂ are defined as above for formulae 3 and 4. In addition, R₅ represents hydrogen, lower alkyl groups, or suitable hydroxyl protecting groups such as methoxymethyl, tetrahydropyranyl, 2-methoxypropyl, 2-methoxyethoxymethyl, triarylmethyl, benzyl, methylthiomethyl, or tert-butyl-dimethylsilyl group. The R₆ group encompasses chemical groups represented by R₁-R₄ as defined above for formulae 3 and 4 as well as substituent cyclohexenylmethyl (5a), limonenyl (5b), and carvone-derived diol acetonide (5c) groups. Other compounds may also be derived from



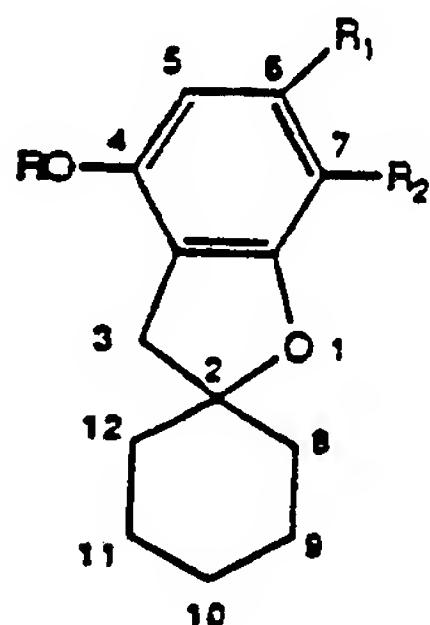
-19-

intermediates 5 and 5a-c, which are, in turn, converted to products of general formulae 3 or 4 as discussed in the following sections. Table V lists representative compounds which comprise the general formula 4 of the present invention; this list is not intended to be comprehensive.

TABLE V

COMPLEMENT INHIBITORS OF THE PRESENT INVENTION*

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15

R = -H, -CH₃, a) - i) from scheme 4
 R₁ or R₂ = -H, -CHO, -COOH,
 -CH₂OH, -CONHCH₃,
 -CONH^tBu, -COCF₃,
 -X (where X = halogen),
 -CH=CHCOOH

4

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* Substituents on the spirocyclohexane ring may also be present; e.g., 10-isopropenyl.

25

The substituted dihydrobenzofuran and spiro-
 benzofuran-2(3H)-cyclohexane compounds of the present
 invention of the general formulae 3, 4, the synthetic
 intermediates of the general formula 5, and the salts
 thereof exhibit complement inhibition, as manifested by
 inhibition of complement-mediated C5a production and/or
 30 inhibition of complement-mediated hemolysis. The comple-
 ment-inhibitory properties of the compounds of the inven-
 tion can be evaluated by modification of known techniques,
 e.g., the assay described in Section 6.6.1, infra.

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Treatment of the compounds of the present invention with appropriate basic reagents provides pharmaceutically acceptable salts thereof. When a carboxylic acid group is present in the compounds of the invention, a pharmaceutically acceptable ester can also be prepared by treatment with a suitable esterifying group under appropriate conditions.

5.2. SYNTHETIC PROCESSES

10 Processes are provided which comprise chemical steps for the synthesis of the compounds of the invention.

Such processes of the invention are diagrammed in detail in Scheme 1 (See infra Section 5.2.2). The synthetic scheme depicted in Scheme 1 provides a shorter and 15 more flexible route than one based on the syntheses of K-76 (Corey and Das J. Am. Chem. Soc. 1982, 104, 5551; McMurray et al. J. Am. Chem. Soc. 1985, 107, 2712). Retro-synthetic evaluation of all the final compounds produced according to Scheme 1 ultimately results in two fragments; 20 an aliphatic and an aromatic portion. These two portions can be joined by using regioselective ortho-lithiation and subsequent alkylation. Two different strategies can then be employed for the production of the final compound of the invention: initial cyclization followed by aromatic 25 functionalization or vice versa.

5.2.1. PREPARATION OF COMPOUNDS OF GENERAL FORMULA 5

The intermediates of the general formula 5 of the 30 invention can be prepared by various methods depending on the types of substituents present therein.

The aliphatic substituent, R₆, of compounds of the general formula 5 are derived from suitable alkylating agents. For example, 1-bromomethylcyclohexene, 6a, can be 35 used as the starting material for the preparation of

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compound 5a. Compound 6a can in turn be prepared from cyclohexanone or directly from alkyl cyclohexene-carboxylate, according to a known process (Wheeler, O.H. and Lerner, I. J. Am. Chem. Soc. 1956, 78, 63; Adams, R. 5 and Thai, A.F. Org. Synth. 1932, 1, 270; Lythgoe, B. et al. J. Chem. Soc. 1956, 406). In a particular example, cyclohexanone is converted to its cyanohydrin, then dehydrated to the cyanoalkene, followed by alcoholysis to an alkyl cycloalkenecarboxylate. Lithium aluminum hydride 10 reduction of the ester followed by halogenation with phosphorus trihalide provides compound 6a.

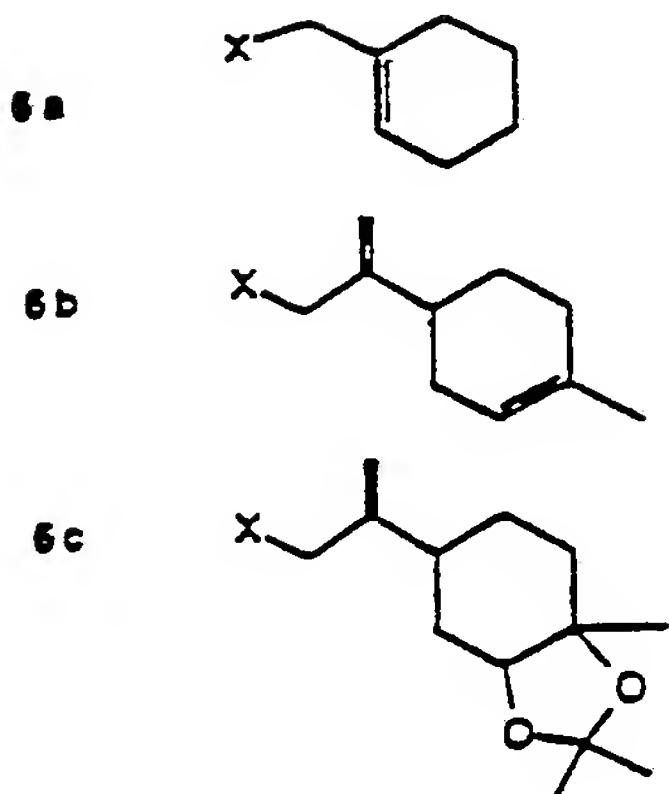
Limonenyl chloride, 6b, is obtained readily from limonenyl alcohol by the action of triphenylphosphine in excess halogenated solvent. This conversion provides 15 optically active allylic halide from optically active limonenyl alcohol. A procedure analogous to that developed by Crawford and co-workers (J. Am. Chem. Soc. 1972, 94, 4298) is used to prepare optically active alcohol by the sequential lithiation of commercially available homo-20 chiral limonene, oxygenation, and reduction of the resultant hydroperoxide with aqueous sodium sulfite.

A third halointermediate, 6c, is derived from a multistep sequence starting from optically active R-(-)- or S-(+)-carvone. The steps of the synthesis involve 25 reduction of the α,β -unsaturated ketone to the allylic alcohol, epoxidation, reduction to the diol, acetonide formation, and treatment of the unsaturated acetonide with calcium hypochlorite to yield the allylic chloride. The coupling reaction is generally carried out immediately 30 after purification of these unstable allylic halides.

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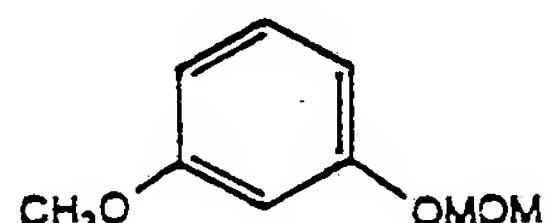
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The aromatic segment of the compounds of the general formula 5 is obtained from substituted alkoxyphenols or resorcinols. For example, 3-methoxymethoxyanisole, 7, is obtained from the reaction of 3-methoxyphenol with chloromethyl methyl ether in a stirred suspension of anhydrous potassium carbonate in acetonitrile (Rall, G.J. H. et al. Tet. Lett. 1976, 1033). Anhydrous conditions and the proper ratio of substrate to solvent are critical to the success of this reaction. For reactions employing quantities of reagents above 5 grams, a useful alternative procedure uses the preformed sodium aryloxide, chloromethyl methyl ether, and dimethylformamide as solvent (See, Rall, G.J.H. et al. supra).

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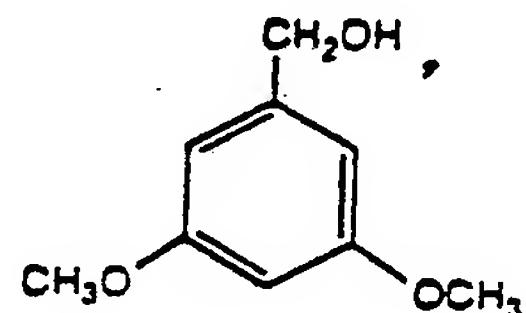
The group R of formulae 5 and 5a-c should be stable to cleavage under the conditions used to hydrolyze or

-23-

remove the protecting group R_5 and the subsequent cyclization of the resulting free phenol according to steps 3 or 4 of Scheme 1. In addition, R_5 is preferably a potentially chelating group which can promote the regioselective ⁵ortho-metalation required to introduce substituents R_2 and R_6 of formula 5. Compound 7 embodies the preferred protecting groups for resorcinol (R = methyl and R_5 = methoxymethyl). The methoxymethyl or MOM ethers can be easily removed in the presence of methyl ethers (Narasimhan, N.S. et al. Synthesis 1979, 906), and the ortho directing power of the MOM group has been shown to be greater than that of a methyl ether (Ronald R.C. Tet. Lett. 1975, 3973).

In an alternative embodiment, the commercially available compound 3,5-dimethoxybenzyl alcohol, 8, can be used ¹⁵ as the aromatic segment for coupling to the allylic halide (See Scheme 2, infra).

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The efficacy of the coupling reaction between the ³⁰ metalated aromatic segment and the aliphatic group is greatly influenced by a number of factors including the degree of aggregation of the metalating species, typically an alkyllithium reagent (Gschwend, H.W. and Rodriguez, H.R. Org. React. 1979, 26, 1). The choice of solvent and any additives such as N, N, N', N'-tetramethylethylenediamine (TMEDA) or hexamethylphosphoric triamide (HMPA), ³⁵

in turn, strongly influences this aggregation state. Solvent(s), additive(s), temperature, reaction time, and reagent stoichiometries for coupling the respective substrates should be optimized. Suitable solvents include 5 anhydrous aprotic organic solvents such as tetrahydrofuran (THF), diethyl ether (Et_2O), dioxane, and diglyme. Surprisingly, we have discovered that dry hexane is a preferred solvent for selective metalation ortho to a chelating group like OMOM and lithioalkoxymethyl (kinetic conditions), while THF/TMEDA solvent mixtures, generally, provide for metalation at the thermodynamic site, between the two inductively electron-withdrawing methoxy groups in 8, for example. In one particular embodiment, n-butyllithium (n-BuLi) in THF/TMEDA or hexane/TMEDA 15 allows for the coupling of aromatic segment 7 with allylic bromide 6a; however, only THF/TMEDA allows coupling of 6a with aromatic segment 8 at the desired 4-position (thermodynamic). When the coupling between 6a and 8 is carried out in hexane, lithiation and coupling occurs in the 20 2-position of 8 (the kinetically favored position).

The aromatic substrate is dissolved in the solvent such as dry THF and the like. The TMEDA is then added and is preferably approximately equimolar to the amount of alkyllithium used. In a preferred embodiment, the amount 25 of alkyllithium (RLi) slowly added at 0°C is approximately 1.1 equivalents per equivalent of a compound of the type such as 7 and is approximately 2.2 equivalents per equivalent of a substrate such as 8 that has an additional acidic hydrogen. Moreover, the addition of approximately 30 1.2 equivalents of a copper salt per equivalent of the lithiated aromatic substrate is preferred to obtain optimum yields from the coupling reaction. Most sources of copper(I) are suitable including cuprous bromide dimethylsulfide, tetrakis (acetonitrile) copper(I) tetrafluoro-

-25-

borate, and the cuprous halides. The use of cuprous iodide is preferred.

Careful control of temperature is important for the success of the aromatic alkylation. While the lithiation 5 is carried out at zero degree to ambient temperature, the subsequent reactions are carried out at low temperature. In a preferred example, the aryllithium reagent is cooled to -78°C followed by the addition of copper(I) salt. The resulting mixture is subsequently stirred at -40°C, a 10 temperature at which the corresponding arylcuprate species can form and is relatively stable. The copper reagent is then recooled to -78°C prior to the addition of the electrophile. Carrying out the the addition at higher temperatures can diminish the yields of coupled products such as 15 5. It is important that all manipulations discussed supra be performed in the absence of oxygen and moisture, and are preferably carried out under an inert atmosphere such as dry nitrogen or argon.

The product mixture which is obtained from the coupling reaction can then be worked up by washing several 20 times with a moderately basic aqueous solution, e.g., saturated aqueous sodium bicarbonate (NaHCO_3). The organic phase can be dried by shaking with a drying agent such as magnesium sulfate, filtered from any solids, and concentrated under a mild vaccum to provide the crude product. 25 Further purification may then be effected by procedures typically employed in the art (e.g., fractional distillation, fractional crystallization, or chromatographic separation).

30

5.2.2. PREPARATION OF COMPOUNDS OF THE GENERAL FORMULAE (3 AND 4)

Substituted dihydrobenzofuran derivatives of the general formula 3 with substituents OR and R_1-R_4 as defined 35 supra in Section 5.1 can be obtained by the metalation and

-26-

subsequent alkylation of coupled intermediates such as those described in the preceding section, followed by hydrolysis of the protecting groups and cyclizaiton of the ene-phenol. This synthetic pathway is illustrated in 5 Scheme 1, infra.

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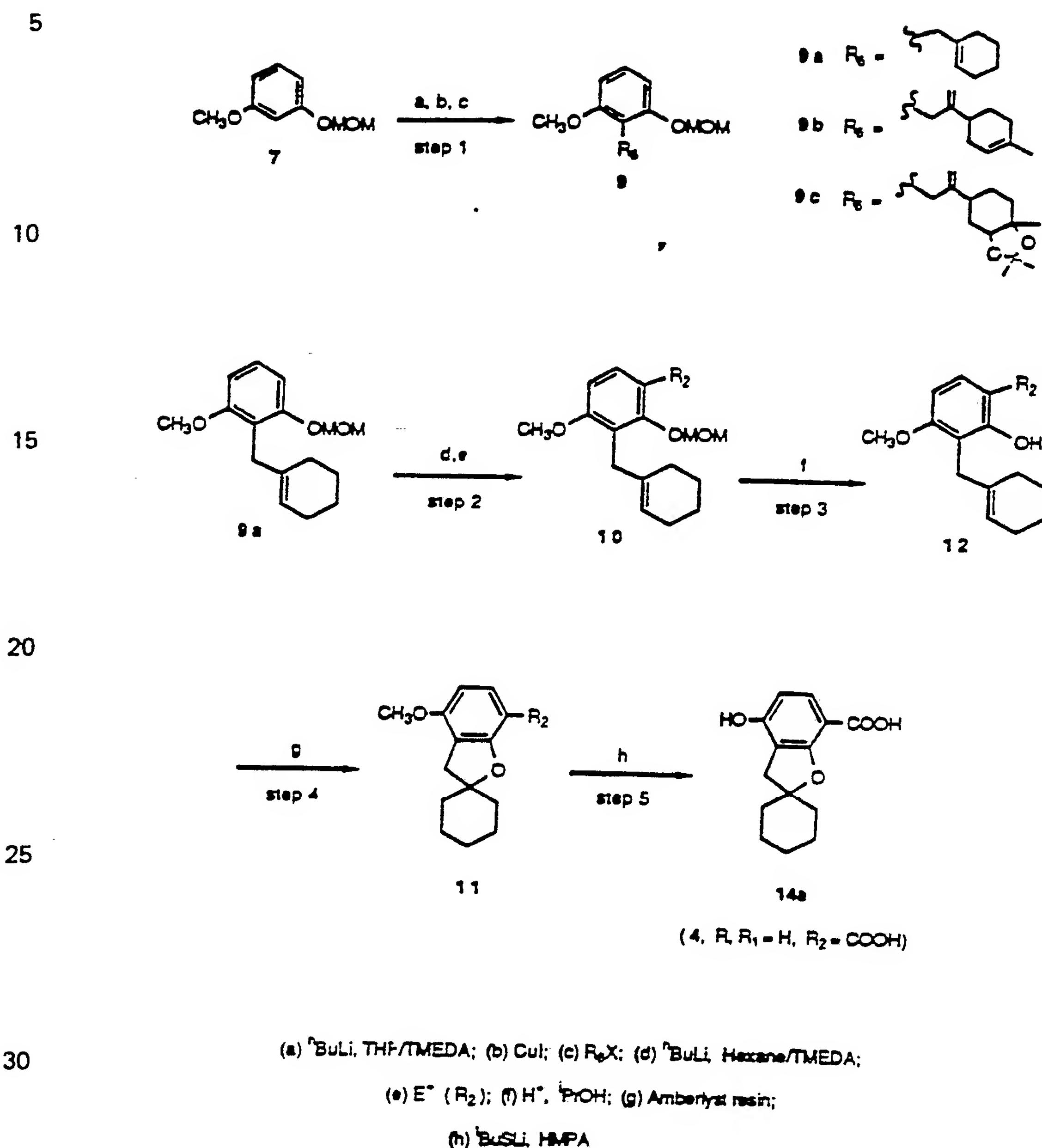
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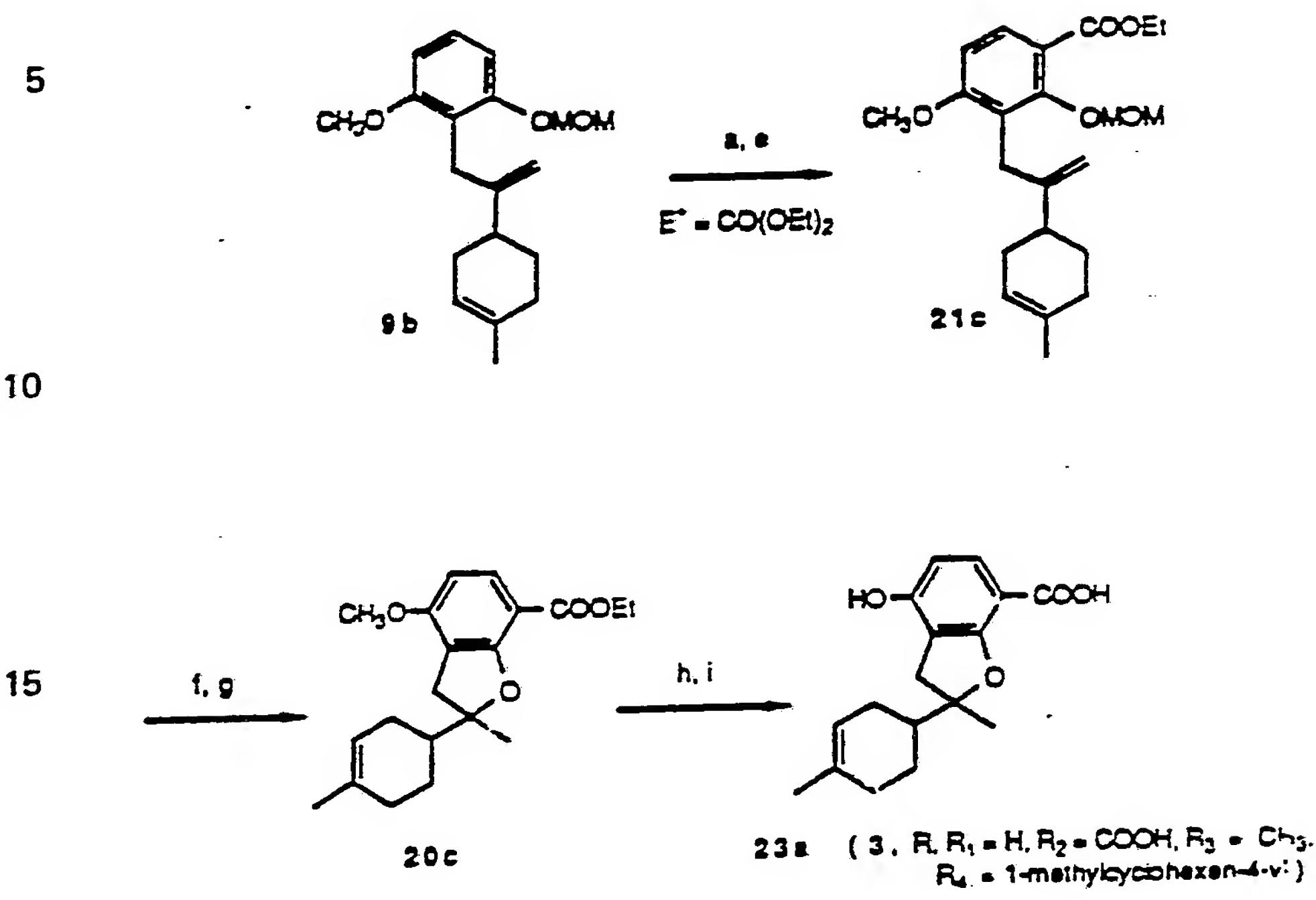
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Scheme 1

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Scheme 1 (cont'd)(a) $^n\text{BuLi}$, THF/TMEDA; (b) Cu^+ ; (c) R_2X ; (d) $^n\text{BuLi}$, Hexane/TMEDA;(e) E' (R_2); (f) H^+ , PrOH ; (g) Amberlyst resin;(h) $^n\text{BuLi}$, HMPA; (i) eq 40% KOH

In a preferred embodiment, the protected resorcinol is lithiated and alkylated in step 1 of Scheme 1 using the allylic bromide 6a as the electrophile. The resulting coupled product 9a can then be dissolved in dry hexane and 5 treated dropwise with alkyllithium in the presence of TMEDA (step 2) at 0°C. Cooling the reaction mixture to -78°C and addition of the desired electrophile introduces the R₂ substituent at this stage.

Generally, the MOM protecting group of intermediates 10 such as 9, with or without the R₂ substituent, may be hydrolyzed by stirring the compound in 5% hydrochloric acid (HCl)/ether mixtures overnight. Deprotection to the free phenol may also be effected by a solvent mixture comprised of 4 N HCl/ⁱPrOH, especially for carboxaldehyde 15 containing derivatives, which need to be stirred for several days. The cyclization step can be achieved most conveniently by stirring the substrate in the presence of Amberlyst resin in a non-polar solvent followed by filtration. In this particular process, the resin is washed 20 with fresh solvent, and the filtrates are combined and concentrated to give the desired crude cyclized product in good yield. In the particular case where R₂ = -COOH in intermediate 10a (Scheme 1, step 2, E⁺ = CO₂), the cyclization of the phenol (steps 3 and 4) can be achieved by 25 stirring with Amberlyst resin in benzene for 24-72 hours at room temperature. Some difficulty is encountered in the cyclization of intermediates with hydroxymethyl substituents (e.g., R₂ = -CH₂OH). A different route is preferred for synthesis of cyclized products with this 30 substituent (see below).

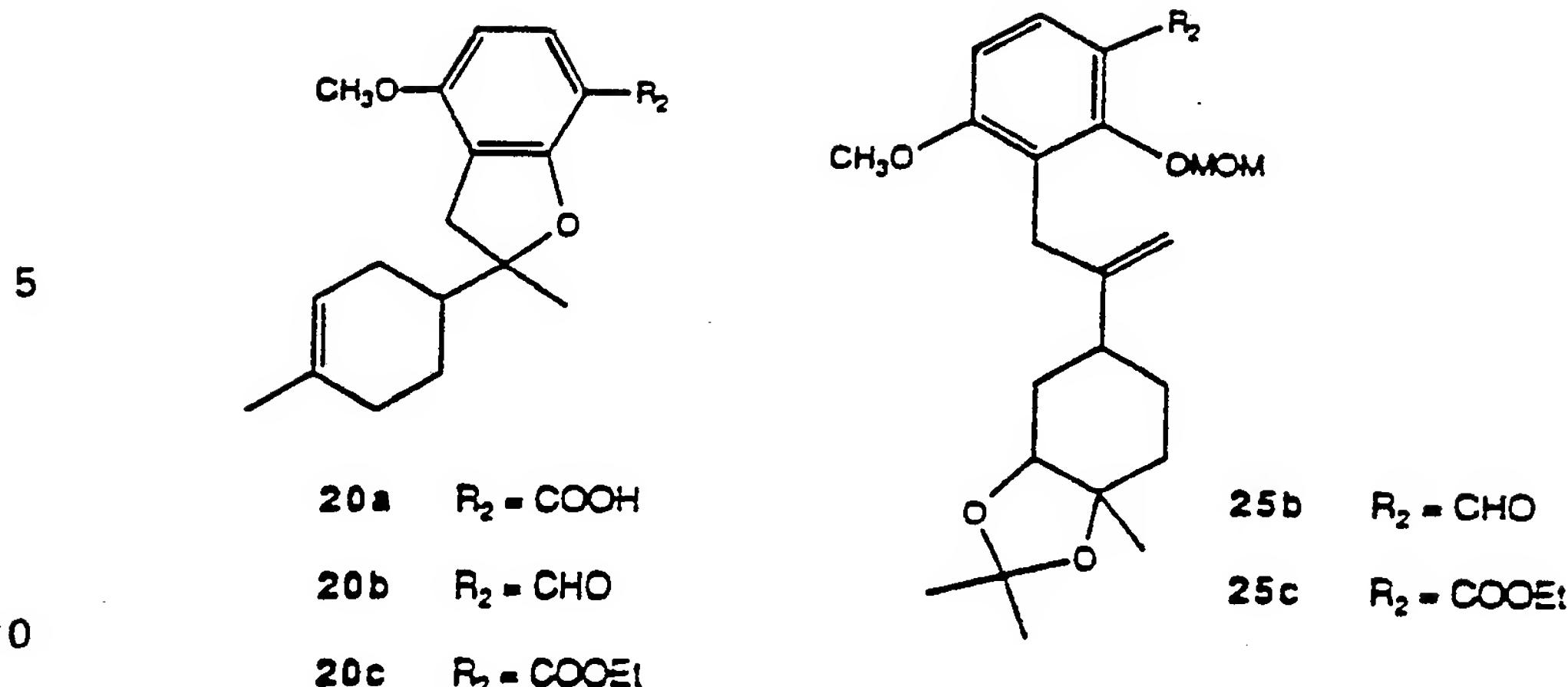
As discussed supra, a variety of R₂ substituents may be introduced before the cyclization step. For example, addition of the following electrophiles to the aryllithium species produces the indicated functional group (these 35

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examples serve only to illustrate a possible technique and are not meant to be limiting): carbon dioxide (carboxyl); ethyl chloroformate or diethyl carbonate (carbethoxy); dimethylformide (formyl); methylisocyanate (N-methyl-
5 carbamoyl); tert-butylisocyanate (N-tert-butylcarbamoyl); paraformaldehyde (hydroxymethyl); trifluoroacetic anhydride (trifluoroacetyl); bromine (bromide); chlorine (chloride); iodine (iodide); substituted vinyl iodides (substituted vinyl group). Other compounds may, in turn,
10 be obtained by modification of these functional groups by known methods.

The cyclized methyl ether derivatives of the type, 11, can be converted to the free phenols, 14 (Scheme 1, step 5), by known methods. These procedures include but
15 are not limited to treatment of the methyl ether with boron trihalides (McOmie, J.F.W. and West, D.E. Org. Synth. 1973, 5, 412; Grieco, P.A. et al. J. Org. Chem. 1975, 40 1450), boron trifluoride in the presence of thiols (Fujita, E. et al. Ibid. 1979, 44, 1661; Fujita, E.
20 J. Chem. Soc. Perkin Trans. 1 1976, 44, 4444), and lithium tert-butylthiolate salts (t-BuSLi) in hexamethylphosphoric triamide (McMurry, J.E. and Erion, M.D. J. Am. Chem. Soc. 1985, 107, 2712). The use of t-BuSLi is preferred.

Open-chain coupled products such as 9b or 9c (See
25 Scheme 1) give rise to compounds such as 21 or 25, respectively. The latter intermediates, wherein the olefinic bond involved in the cyclization is exocyclic, or other derivatives containing simple unsaturated aliphatic groups, produce dihydrobenzofuran derivatives disubstituted at the 2-position upon deprotection and treatment
30 with Amberlyst ion-exchange resin (See e.g., 20).



Slightly more complex analogs containing a diol group, such as 24 or 27, *infra*, can be obtained in this manner.

The use of 3,5-dimethoxybenzyl alcohol, 8, as the starting aromatic component has the advantage of having the R_1 substituent already in place. Compound 8 is dissolved in THF and is treated at 0°C with 2.0 equivalents 20 of *n*-butyllithium in the presence of TMEDA (2.0 equiv). The benzyl alcohol is lithiated predominantly at the 4-position after about 2 hours. Formation of the arylcuprate at -40°C and addition of the alkylating agent completes the synthesis of 13 (Scheme 2, step 1) with a small 25 amount of the isomer 13' also being formed. Fractional crystallization of the product mixture provides pure 13.

As previously alluded to, cyclization of intermediates with hydroxymethyl substituents can be problematic. In one embodiment, a remedy to this problem involves 30 converting the benzyl alcohol 13c to the formyl compound 13b (See Scheme 2, step 2). This procedure can be accomplished by a Swern oxidation or, more preferably, by using pyridinium chlorochromate in "buffered" methylene chloride. One of the symmetrical methyl ethers is then 35 selectively cleaved (step 3) and cyclized in the normal

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fashion to yield the 6-substituted formyl analog **30b**. Oxidation of the formyl group to the carboxylic acid (e.g., **30a** or **31a**) can be effected by a number of reagents either before or after the cyclization of step 4. Suitable inorganic oxidizing agents include but are not restricted to permanganate salts, manganese oxide, chromic acid, chromate salts, silver oxide, silver nitrate, nickel peroxide, and cerium salts such as cerium sulfate, cerium oxide and cerium perchlorate. Silver oxide is preferred. The benzyl alcohol, **30c** (4, R = CH₃, R₁ = CH₂OH, and R₂ = H), is obtained by reduction of **30b** using, for example, such reducing agents as lithium aluminum hydride, or diborane, preferably in an appropriate solvent such as ether, e.g., THF, dioxane, or diethyl ether.

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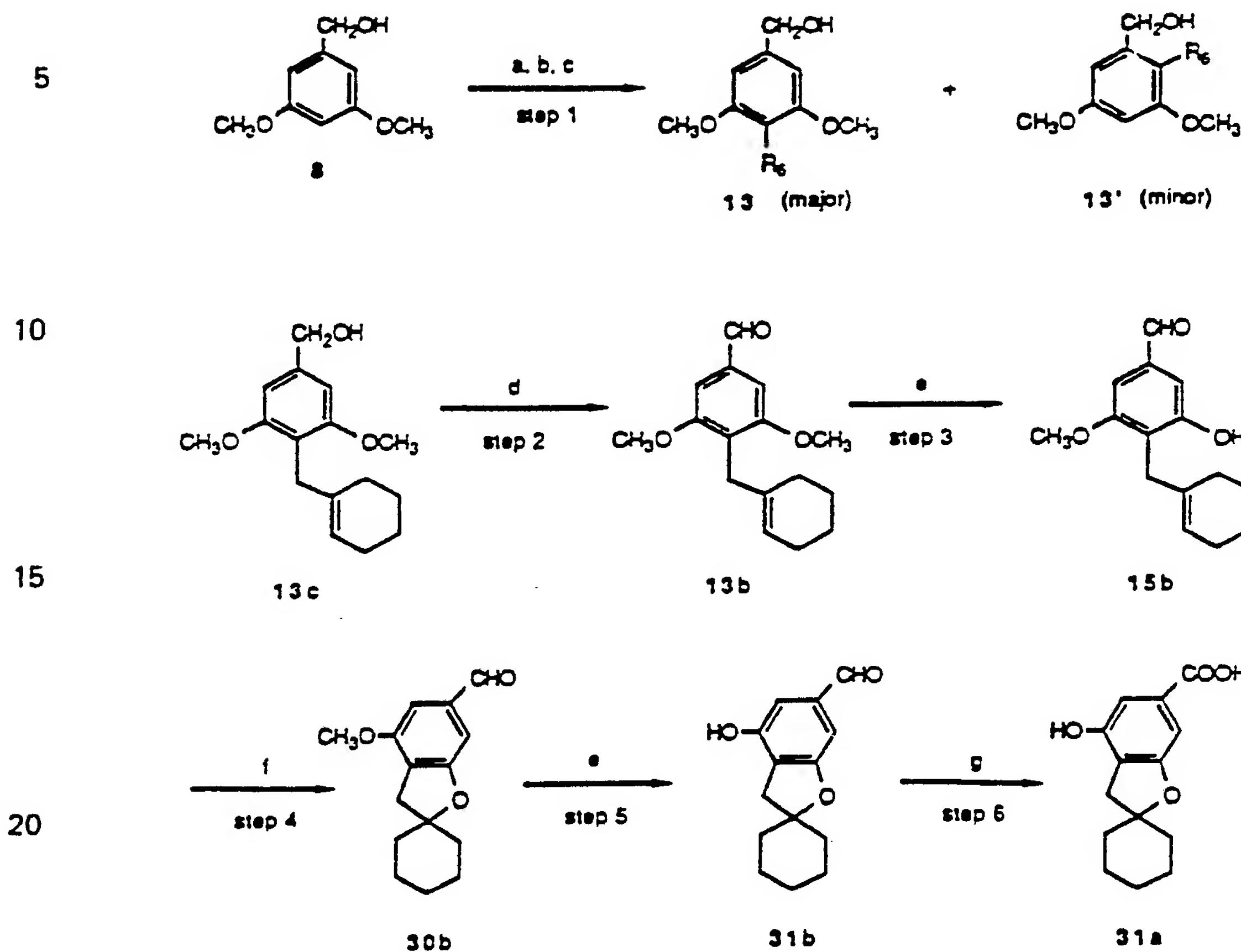
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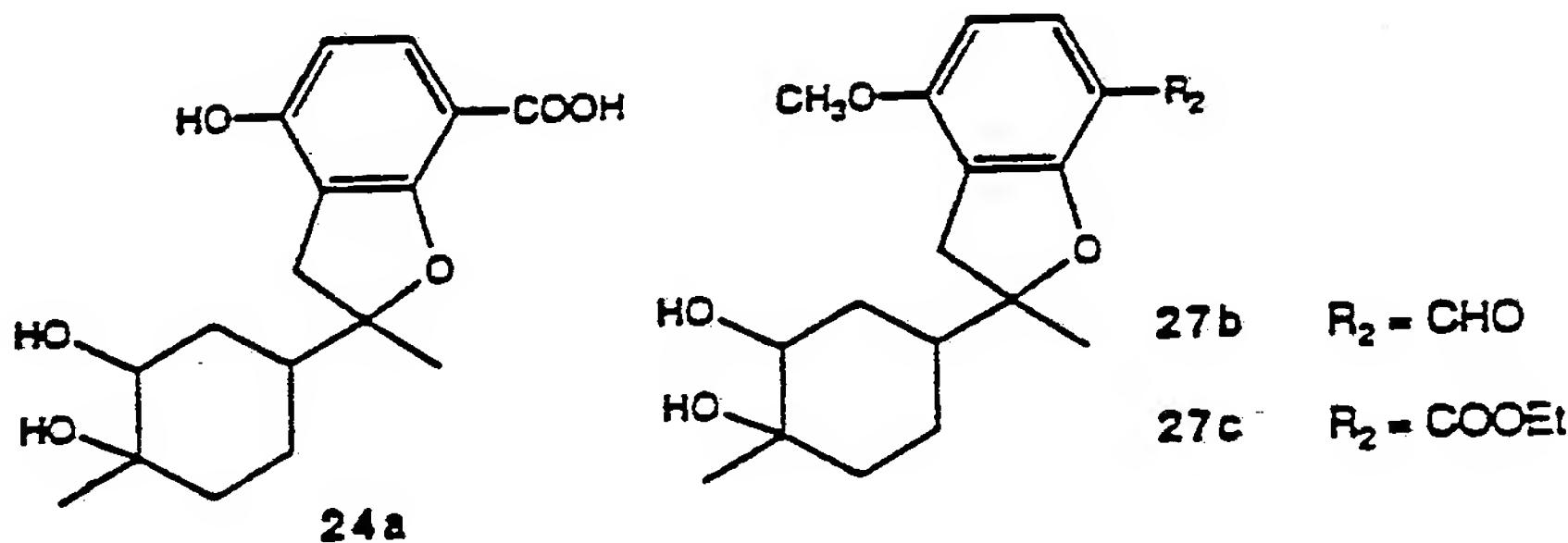
Scheme 2

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Even more complex dihydrobenzofuran-based compounds can be obtained by further modification of the analogs described above. Any of the compounds containing the methyl ether groups at the 4-position of the dihydrobenzo-⁵furan skeleton can be cleaved by one of the boron or thiolate reagents mentioned supra. Sometimes, as in the limonene series, it may be desirable to carry out the demethylation of the methyl ether with the carboxylic acid group at the 7-position protected as its alkyl ester (See, e.g., Scheme 1). Open positions in the phenyl ring may be metalated and functionalized as disclosed previously. The double bond of compound 20a can be hydroxylated by transition metal oxide reagents such as potassium permanganate or osmium tetroxide. The combination of methylether ¹⁰ cleavage and hydroxylation of 20a, for example, would give compound 24a. Alternatively, compound 24a may be obtained by demethylation of intermediate 27c to the free phenol. In those instances, as in here, where an alkyl ¹⁵ phenol.

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ester is employed to protect the carboxylic acid group at the 7-position, an alkaline, hydrolysis step may be necessary to obtain the free carboxylic acid (Scheme 1, step i). Other modifications can be envisioned which do not

-35-

depart significantly from the scope and essence of the products of the present invention. Transformations such as epoxidation of the double bond, hydroxylation at the allylic position of the double bond, oxidate cleavage of 5 the double bond, hydroformylations, and modifications of the products obtained therefrom are but a few non-limiting examples.

5.2.3. PREPARATION OF COMPOUNDS OF GENERAL FORMULA
10 6-CARBOXYL-4-SUBSTITUTED SPIRO[BENZOFURAN
-2(3H)-CYCLOHEXANES]

6-carboxyl spiro[benzofuran-2(3H)-cyclohexane] molecules that are also substituted at the 4 position to form a series of ether substituted derivatives can be prepared as shown in Schemes 3 and 4 below and as more fully described in Section 7 infra.
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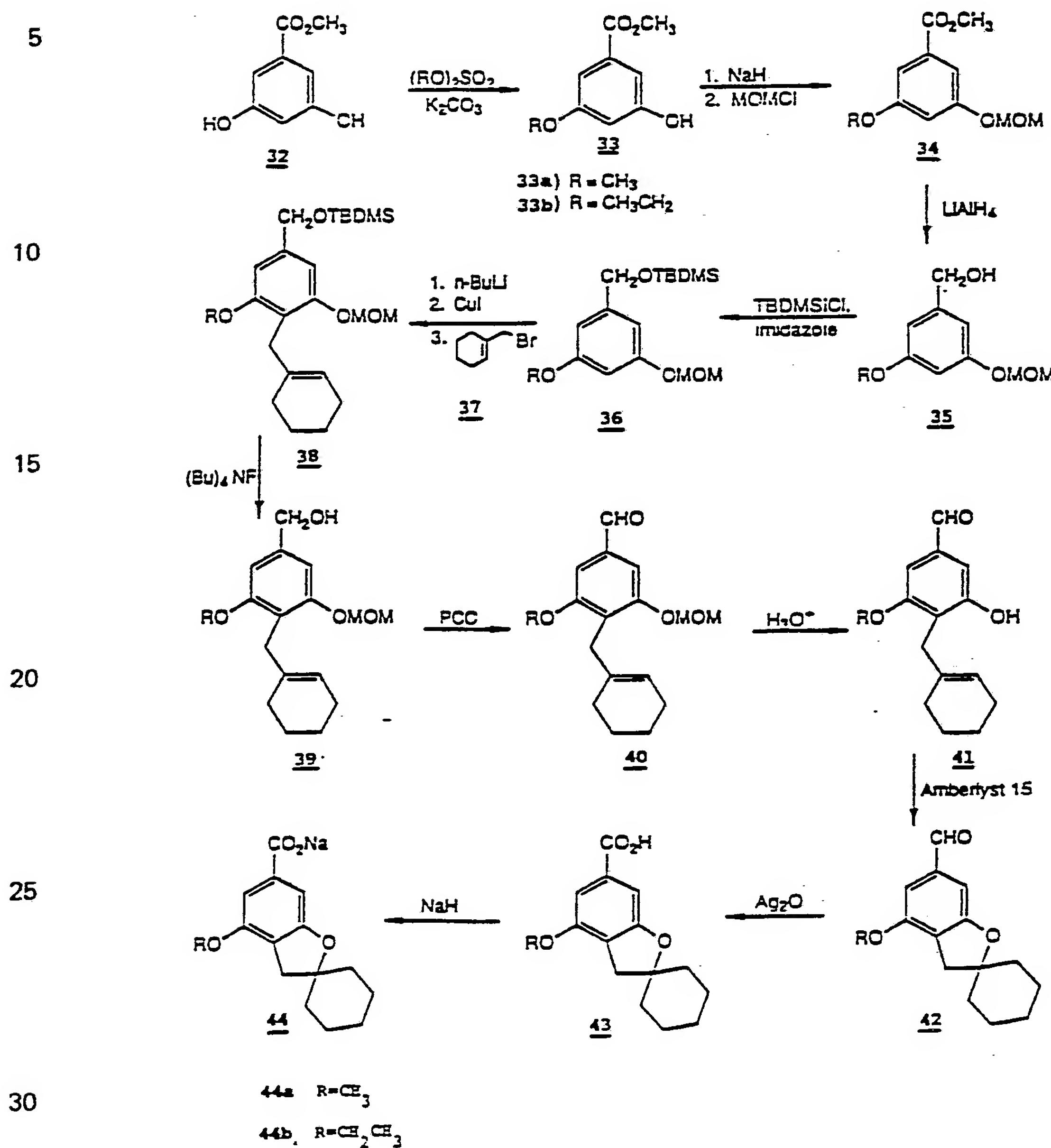
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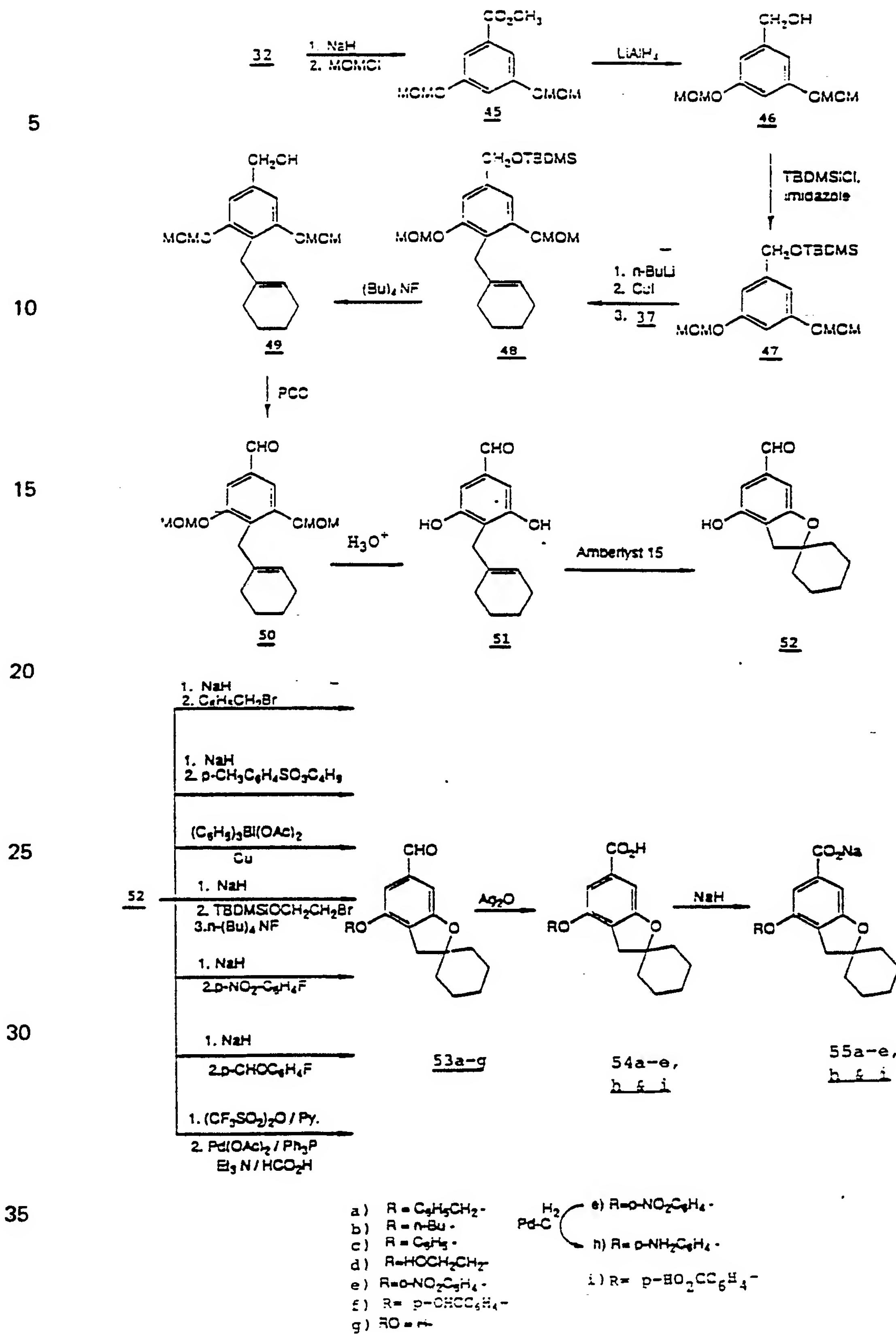
-36-

Scheme 3



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Scheme 4



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These compounds and the salts thereof exhibit complement inhibition as manifested by inhibition of complement mediated hemolysis. Other complement-inhibitory properties of these compounds can be evaluated by known techniques as described in Sections 6.6.1 and 10.2 to 10.4 and by numerous complement assay techniques that are known in the art. Substituents for R in position 4 of compounds of the general formula 4 include a hydrogen atom or a lower alkyl group (e.g. CH₃-, CH₃CH₂-, n-Bu-), a functionalized lower alkyl group (e.g. HOCH₂CH₂-), a benzyl or substituted benzyl group, a phenyl or substituted phenyl group (e.g. C₆H₅CH₂-, C₆H₅-, p-NO₂C₆H₄-, p-CHOC₆H₄-, p-NO₂C₆H₄-, and p-NH₂C₆H₄-). In addition the OR at position 4 of compounds of the general formula 4 can instead be H-. Preferred substitutions at position 4 are exemplified by compounds **44b**, **55c** and **55e**, which are more effective in inhibiting complement mediated hemolysis than is K76COOH.

5.2.4. PREPARATION OF COMPOUNDS OF GENERAL FORMULA
20 6, 7 -DISUBSTITUTED -4-METHOXYSPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANES]

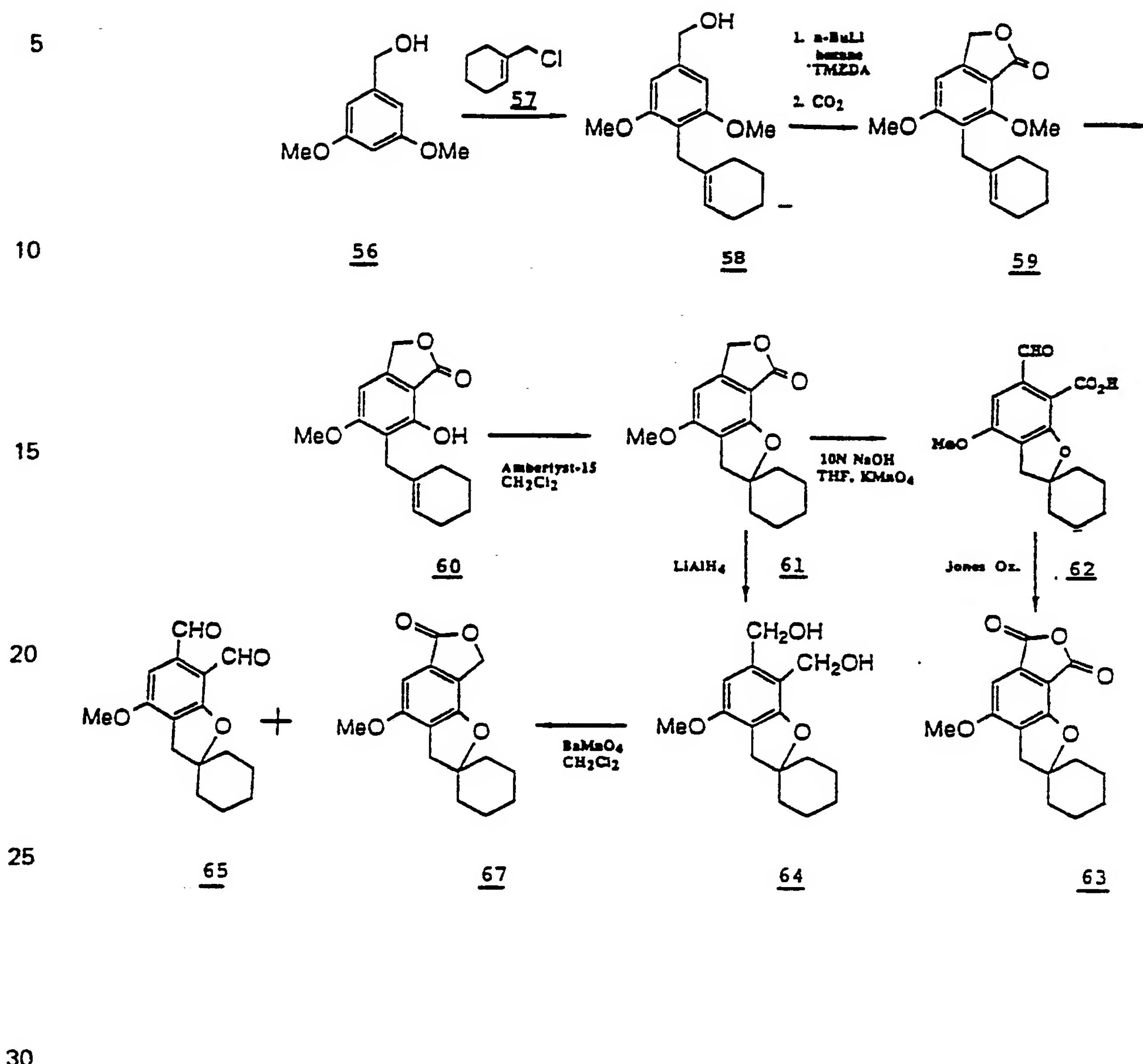
6,7-disubstituted spiro[benzofuran-2(3H)-cyclohexane] molecules can be prepared as shown in Schemes 5 and 6 below and as more fully described in Section 8 infra.

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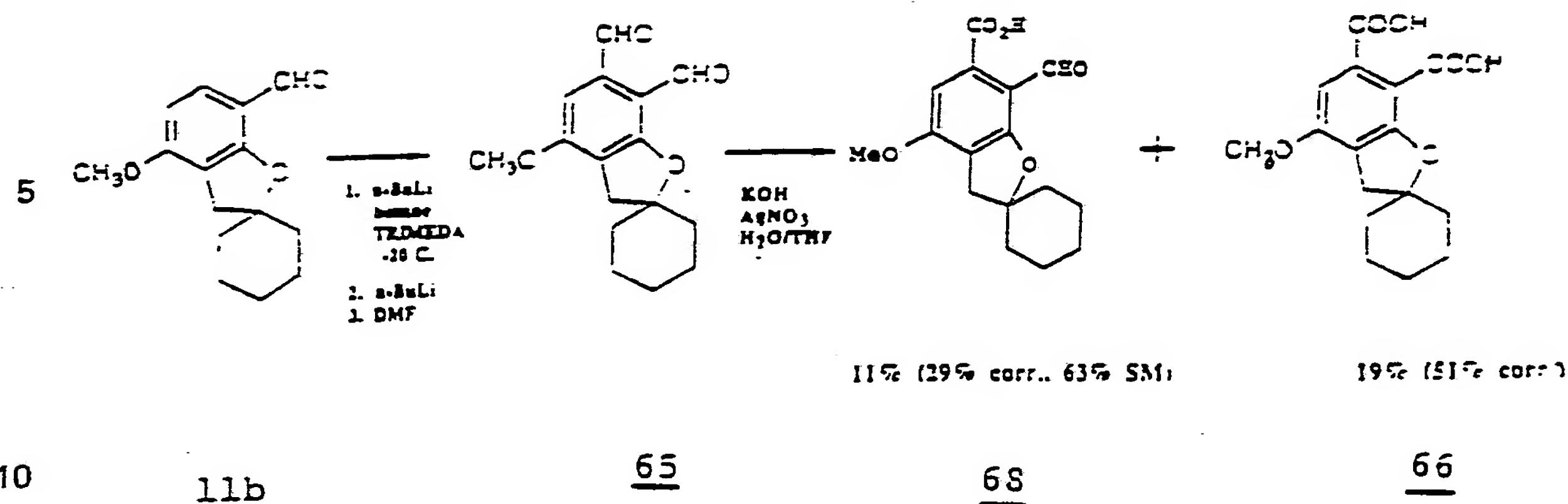
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Scheme 5

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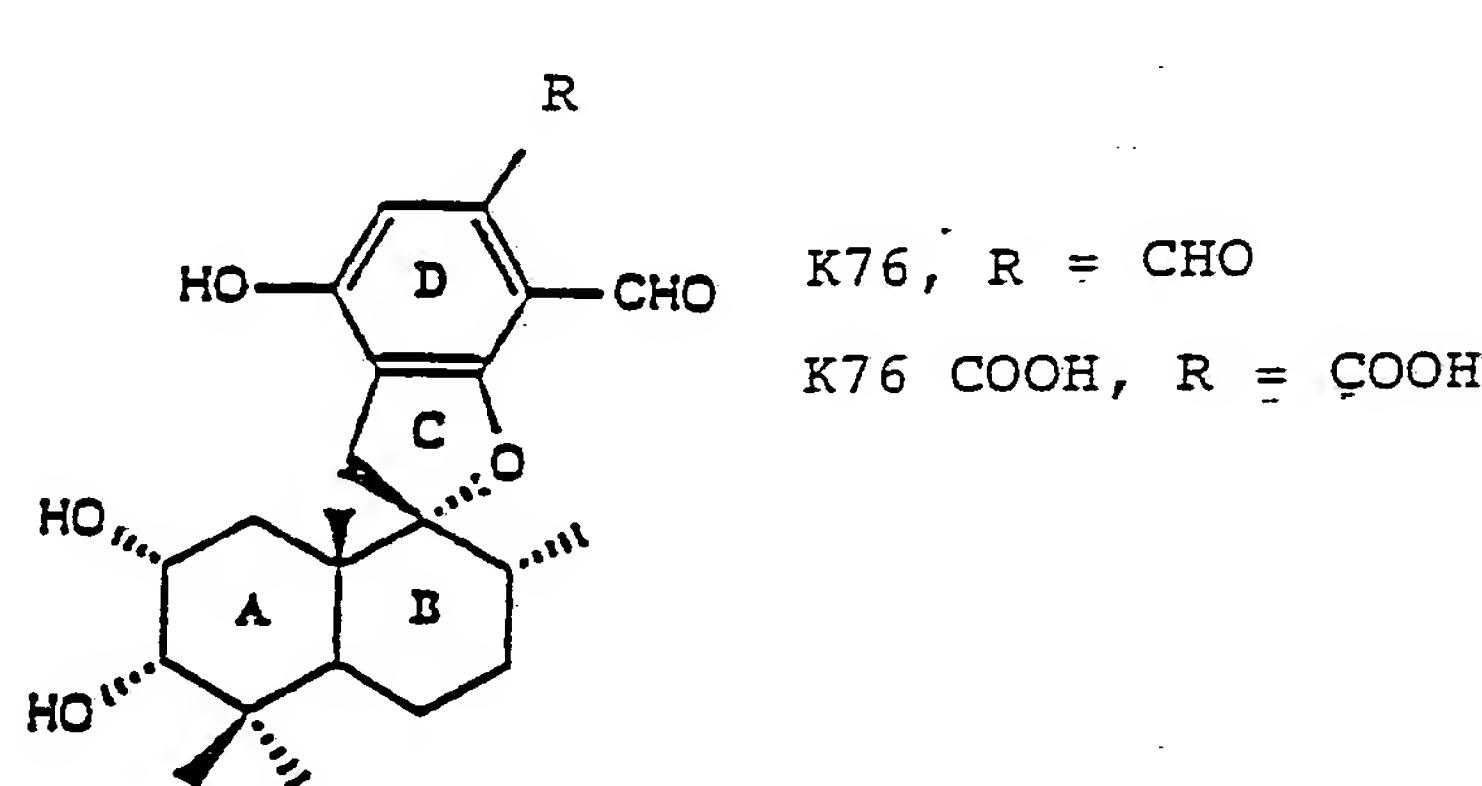
Scheme 6

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The compounds of this section differ from K76, since they represent disubstituted BCD analogues lacking the A ring structure of K76. In addition the compounds of this section can also be substituted at position 4 of the D ring to form trisubstituted BCD analogues. These di- and tri-substituted compounds and the salts thereof exhibit complement inhibition as manifested by inhibition of

complement mediated hemolysis. Other complement-inhibitory properties of these compounds can be evaluated by known techniques as described in Sections 6.6.1 and 10.2 to 10.4 and by numerous complement assay techniques that are known 5 in the art.

The R₁ and R₂ groups can be any combination of the following: a hydrogen atom, a carboxylic acid group, a formyl group, a hydroxymethyl group, an N-(lower alkyl) carbamoyl group, a trifluoroacetyl group, a carbalkoxy 10 group, a halide group, a vinyl group, a substituted vinyl group having up to 10 carbon atoms, an alkylidene group having up to 20 carbons, an aliphatic acyl group, a substituted aliphatic acyl group, an aromatic group, a substituted aromatic acyl group, a trifluoroacetyl group, a 15 sulfamoyl group, an N-acylcarbamoyl group, a tetrazole group, a tertiary aliphatic amine group, an oxazoline group, an amidine group, or a hydrazone group.

Specific substitutions for R₁ and R₂ groups include -CHO, -CH₂OH, -COOH, COCF₃, SO₂NH₂ and tetrazole, oxazo-20 line, imide or CH₂NMe₂ derivatives. Substitutions at positions 6 and 7 can be cyclic compounds as exemplified by compounds 62 and 68. The presence of polar groups in the 6 and 7 positions appears to affect complement inhibition activity, perhaps because such polar groups interact with 25 regions of the complement receptors.

Specific compounds include compounds of general formula 4 in which R can vary generally as described above; R₁ is a carboxyl group or a bioisosteric acid group (such as sulfonamide, imide, or tetrazole) or a bioisosteric basic group (such as a tertiary aliphatic amine, oxazoline, amidine, or hydrazone), or a bioisosteric neutral group (such as trifluoroacetyl); R₂ is a formyl group or a bioisosteric group such as methyl ketone (acetyl), other alkyl ketone, aryl ketone or other similar 30 group, or a bioisosteric group such as a sulfonamide, imide, or tetrazole.

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A preferred organic compound of the compositions of this invention is the 4,6,7 trisubstituted spiro[benzofuran-2(3)H cyclohexane] such as compound 68 (6-carboxy-7-formyl-4-methoxyspiro[benzofuran-2(3H)-cyclo-5hexane]) and 6-carboxy-7-formyl-4-phenoxy-spiro[benzo-furan-2(3H)-cyclohexane]. In a specific embodiment, compound 68 exhibits a relatively large amount of complement inhibition in the hemolysis assay. Even more inhibitory compounds can be produced by combining the optimal substitutions at the 4 position with the 6,7 disubstitutions present in 68. In a preferred embodiment, a phenoxy group is substituted at the 4 position.

5.3. DEMONSTRATION OF COMPLEMENT INHIBITION

15 The compounds of the invention can be assayed by any techniques known in the art in order to demonstrate their complement inhibiting activity. Such assays include but are not limited to the following in vitro tests for the ability to inhibit complement system activity or to selectively inhibit the generation of complement-derived peptides (See Sections 6.6 and 10.3 for specific examples):

- (i) measurement of inhibition of complement-mediated lysis of red blood cells (hemolysis);
- 25 (ii) measurement of ability to inhibit formation of C5a and C5a des Arg and/or measurement of ability to inhibit formation of C3a and C3a des Arg;
- (iii) inhibition of alternative pathway mediated hemolysis.

30 Those compounds which are demonstrated to have significant complement-inhibiting activity can be therapeutically valuable for the treatment or prevention of diseases or such as those described in Section 5.5, infra.

5.4. IMMUNOSUPPRESSIVE ACTIVITY

The compounds of the present invention can inhibit immune activity. In particular, the compounds of the invention inhibit cell-mediated immune function. For example, the compounds can suppress natural killer activity, inhibit the proliferation of peripheral blood lymphocytes, and/or inhibit the activation of T lymphocytes in PBL culture.

Any procedure known in the art may be employed to demonstrate immunosuppressive activity. Such procedures include but are not limited to in vitro assays for inhibition of natural killer lysis of target cells, inhibition of proliferation of peripheral blood lymphocytes or inhibition of cell surface interleukin-2 receptor expression. Specific embodiments of assay procedures which can be used are detailed in the examples sections infra (See Subsections 6.7.1 through 6.7.4).

20 5.5. THERAPEUTIC USES OF THE COMPOUNDS OF THE INVENTION

The compounds of the invention which exhibit complement and/or immune activity inhibition have therapeutic value in the prevention or treatment of various immune or inflammatory diseases or disorders. The compounds of the invention may be administered to a patient for treatment of an immune disorder involving undesirable or inappropriate complement activity. In particular, an effective dose of an inhibitive compound of the invention may be therapeutically applied to ameliorate or to prevent a detrimental effect caused by the activity of a component of the complement system (e.g., C5a) or an inappropriately reactive immune system. An effective dose of the compound of the invention for the treatment of a disorder involving undesirable or inappropriate complement activity or an immune disorder can be determined by standard means known

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in the art taking into account routine safety studies, toxicity studies, dose concentration studies and method of delivery, e.g., bolus, continuous or repeated. In a particular embodiment, a dose of about 0.01 to about 500 5 mg/kg can be administered.

The diseases or disorders which may be treated by the compounds of the invention include but are not limited to those listed in Table III, supra. In particular, those disorders associated with extended zones of tissue destruction due to burn- or myocardial infarct-induced 10 trauma, and adult respiratory distress syndrome (ARDS), also known as shock lung, can be treated by administration of an effective amount of the compounds.

Detrimental nonspecific activation of the complement 15 system, or unfavorable activation by the alternative pathway, can also be prevented or treated by compounds of the invention. In specific embodiments, such compounds can ameliorate the acute pathological changes induced by specific or non-specific proteolytic processing of C5.

20 The compounds of the invention may also be used to modulate biologic or immune functions directly or indirectly mediated by the complement system, which can include but are not limited to those functions listed in Tables I and II, supra, and the in vivo correlates of the 25 in vitro functions therein.

In particular embodiments, the inhibitive compounds can be used to treat inflammation associated with, for example, kidney stones, systemic lupus erythematosis (SLE), nephrotoxic glomeronephritis, or multiple sclerosis 30 (See, e.g., Experimental Allergic Encephalomyelitis. A Useful Model for Multiple Sclerosis, A Satellite Conference of the International Society of Neurochemists, July 16-19, 1983, University of Washington, Seattle, Washington; Miyazaki, W. et al. Microbiol. Immunol. 1980, 24,

-45-

1091; Konno, S. and Tsurutuji, S. Sr. J. Pharmacol. 1983,
80, 269).

In yet another embodiment, the compounds of the invention can be administered for treatment of tissue damage due to myocardial ischemia and reperfusion, resulting from neutrophils attracted by and activated by the complement system.

The compounds of the invention may also be administered for the prevention or treatment of diseases or disorders caused or accompanied by increased lymphocyte or natural killer activity, including but not limited to atrophic gastritis, thyroiditis, allergic encephalomyelitis, gastric mucosa, thyrotoxicosis, autoimmune hemolytic anemia, pemphigus vulgaris, sympathetic ophthalmia, delayed-type hypersensitivity, rejection of allografts, graft-host reaction, organ transplant rejection, other autoimmune disorders, and drug allergies. They can also be used to alleviate the adverse effects of complement activation caused by therapeutic intervention such as tissue plasminogen activator therapy or cardiopulmonary bypass.

Pharmaceutical compositions comprising the inhibitive compounds or the salts thereof are provided by the present invention. Such compositions comprise a therapeutically effective amount of the compound and a pharmaceutically acceptable carrier. Such a carrier includes but is not limited to saline, buffered saline, dextrose, and water. A pharmaceutical kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical composition is also within the scope of the invention.

Various delivery systems (e.g., encapsulation in liposomes, microparticles, or microcapsules, conjugation to specific molecules) are known and can be used for therapeutic delivery of the compounds. Methods of admin-

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istration include but are not limited to oral, intradermal, transdermal, intravenous, subcutaneous, intramuscular, intraperitoneal, and intranasal routes. Such administration can be done in either bolus or repeat doses or 5 continuously by infusion for instance.

A further embodiment of this invention includes the combined therapy that can be obtained by treating patients with disorders (e.g. myocardial infarction patients) that are routinely treated with thrombolytic agents such as 10 tissue plasminogen activator, streptokinase or urokinase with a combination of the compounds of this invention and the routinely administered thrombolytic compounds or a fibrinolytically active fragment, derivative, or modified version thereof. The usefulness of such a combined therapy derives from the observation that the complement system 15 is activated in disorders such as myocardial infarction or bypass surgery. The efficacy of a combined treatment could be substantially better than the thrombolytic treatment alone due to the ability of the complement inhibitory 20 compounds to modulate the inappropriate and damaging complement activation. The administration of the thrombolytic and complement inhibitory compounds can be simultaneous or sequential or in different dose forms including combinations of oral dose forms with injectables to name 25 just a few.

The invention can be better understood by referring to the following examples which are given for illustrative purposes only and are not meant to limit the invention.

30

6. EXAMPLES

6.1. 2-(1'-CYCLOHEXENYL)METHYL-3-METHOXYMETHOXYANISOLE (9a)

The 3-methoxymethoxyanisole, 7, was obtained in 96% yield by the following procedure. A mixture of finely 35 powdered anhydrous potassium carbonate (K_2CO_3 ; 2.0 equiv)

and 3-methoxyphenol (1.0 equiv) in dry acetonitrile was stirred at 0°C for 15 minutes under nitrogen. To ensure that the reaction pH remained above six, 100 ml of acetonitrile were used per gram of phenol substrate. A catalytic amount of 18-crown-6 (0.12 equiv) was added, and the mixture was stirred an additional 15 minutes at 0°C. Neat chloromethyl methyl ether (1.5 equiv) was then introduced slowly. The suspension was allowed to warm up to ambient temperature and stirred for 6 hours. After this time, the mixture was recooled to 0°C, and one-half the original quantities of K₂CO₃, 18-crown-6, and CH₃OCH₂Cl were added. After another 4 hours of stirring at room temperature, the suspension was filtered and the filtrate was concentrated under reduced pressure. The residue was dissolved in ethyl ether (Et₂O), washed with 5% sodium hydroxide (3 x 50 ml), concentrated in vacuo, and distilled under reduced pressure. Compound 7 was obtained as a clear colorless liquid. Bp 45°C (0.15 mm Hg). ¹H NMR (90 MHz, deuteriochloroform) δ 7.16 (1H, m), 6.61 (3H, m), 5.16 (2H, s), 3.79 (3H, s), and 3.49 (3H, s) in ppm downfield from TMS. ¹³C NMR (CDCl₃) δ 160.5, m 158.2, 129.7, 108.2, 107.3, 102.5, 94.3, 55.9, and 55.1 in ppm downfield from TMS.

n-BuLi (1.1 equiv) was added slowly to a THF solution of compound 7 (1.0 equiv) and TMEDA (1.1 equiv), at 0°C under a nitrogen atmosphere. The solution was stirred at room temperature for 2-5 hours and then cooled to -78°C. Cuprous iodide (1.2 equiv.) was added all at once. The light gray suspension was warmed up to -40°C, and after stirring for 1.5 hours, turned into a green-gray color. The copper reagent was cooled to -78°C and allowed to react with a THF solution of freshly-prepared allylic bromide 6a (1.3 equiv). The reaction mixture was allowed to warm up to ambient temperature gradually and stirred for up to 72 hours. The mixture was quenched and washed

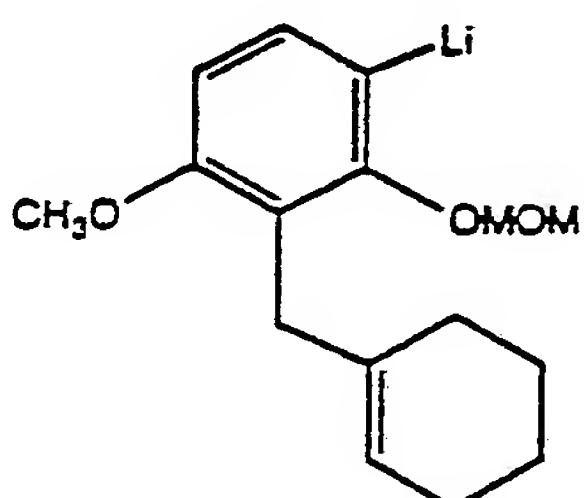
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with a saturated aqueous solution of sodium bicarbonate until the aqueous layer became colorless. The organic layer was dried by passage through a plug of potassium carbonate and concentrated in vacuo to give a dark orange oil. The crude product was distilled under reduced pressure to provide compound **9a** in 69% yield. Bp 105°C (0.15 mm Hg). Anal. Calcd. for C₁₆H₂₂O₃: C, 73.25; H, 8.45. Found: C, 73.13; H, 8.50. ¹H NMR (90 MHz, CDCl₃) δ 7.03 (1H, t, J = 8 Hz), 6.71 (1H, d, J = 8 Hz), 6.54 (1H, d, J = 8 Hz), 5.23 (1H, broad s), 5.13 (2H, s), 3.77 (3H, s), 3.43 (3H, s) 3.32 (3H, broad s), 1.95 (4H, m), and 1.57 (4H, m) in ppm downfield from TMS. ¹³C NMR (75 MHz, CDCl₃) δ 158.5, 155.8, 136.3, 126.7, 120.0, 118.1, 107.0, 104.6, 94.3, 55.8 (2C's), 30.9, 28.8, 25.3, 23.1, and 22.6 ppm downfield from TMS. IR (neat) 2940, 2840, 1595, 1470, 1440, 1260, 1160, 1105, 1070, and 1025 cm⁻¹.

6.2. 3-(1'-CYCLOHEXENYL)METHYL-2-HYDROXY-4-METHOXYBENZOIC ACID (**12a**)

Compound **9a** was metalated at the 4-position by the following procedure. A hexane solution of **9a** (1.0 equiv) and TMEDA (1.1 equiv) was treated with a hexane solution of n-BuLi (1.1 equiv) added gradually at 0°C under an atmosphere of nitrogen. The solution was stirred at room temperature for 3 hours and then cooled to -78°C.

30



Li-9a

The aryllithium reagent **Li-9a** prepared by the above route was then exposed to a stream of dried carbon dioxide

gas bubbled through the -78°C solution for 0.5 hour. Carrying out this reaction at 0°C cuts the resulting yield in half. The mixture was allowed to warm up to room temperature while maintaining a steady stream of gas. The 5 mixture was poured into water and extracted a few times with 5% aqueous sodium hydroxide, then acidified to pH 1 with concentrated hydrochloric acid, and then extracted into ether. The crude product was back-extracted into Et₂O and the combined organic layers dried over magnesium 10 sulfate. The solvent was evaporated and the residue redissolved in a minimum of boiling ether. The warm solution was allowed to cool slightly, and then hexane was added to the point of cloudiness. The mixture was then allowed to stand in the freezer. An off-white solid with 15 a melting point of 161-163°C was harvested (66% yield).

Anal. Calcd. for C₁₅H₁₈O₄: C, 68.69; H, 6.92. Found: C, 68.75; H, 6.95. ¹H NMR (90 MHz, acetone-d₆) δ 7.81 (1H, d, J = 9 Hz), 6.62 (1H, d, J = 9 Hz), 5.26 (1H, broad s), 4.26 (2H, broad s), 3.90 (3H, s), 3.29 (2H, broad s), 2.01 20 (4H, m), and 1.57 (4H, m) in ppm downfield from TMS. ¹³C NMR (acetone-d₆) δ 173.0, 164.3, 162.1 (2C's), 136.5, 130.6, 121.0, 115.9, 106.5 (2C's), 103.3, 56.3, 30.0, 29.4, 25.8, 23.8, 23.2 (2C's). IR (KBr) 1650, 1610, 1500, 1455, 1265, 1185, and 1090 cm⁻¹.

25

6.3. 3-(1'-CYCLOHEXENYL)METHYL-2-HYDROXY-4-METHOXYBENZALDEHYDE (12b)

A solution of aryllithium reagent Li-9a, prepared in situ by the procedure described in Section 6.2, was cooled 30 to -12°C and treated with neat N,N'-dimethylformamide (1.5 equiv) added all at once. The mixture was stirred at room temperature for 3 hours. The mixture was then poured into water, saturated with sodium chloride, and extracted with Et₂O. The organic layers were combined, dried (MgSO₄), and 35 concentrated in vacuo; the residue was recrystallized from

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ether-hexane (See Section 6.2). The product was purified by column chromatography (silica, ether/hexane eluent). Mp 48-49°C. Anal. Calcd. for C₁₅H₁₈O₃: C, 73.15; H, 7.37. Found: C, 73.22; H, 7.40. ¹H NMR (CDCl₃) δ 11.42 (1H, 5 broad s), 9.64 (1H, s), 7.33 (1H, d), 6.51 (1H, d), 5.23 (1H, broad s), 3.83 (3H, s), 3.26 (2H, broad s), 1.96 (4H, m), and 1.56 (4H, m) in ppm downfield from TMS. ¹³C NMR (CDCl₃) δ 194.7, 164.6, 161.3, 135.5, 133.8, 120.6, 115.9, 115.6, 103.1, 55.9, 29.9, 28.8, 25.2, 23.0, and 22.4 in 10 ppm downfield from TMS. IR (KBr) 2930, 2840, 1625, 1495, 1255, 1100, 800, and 640 cm⁻¹.

6.4. 7-CARBOXY-4-METHOXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (11a)

15 A benzene solution of compound 12a (Section 6.2) was treated with dry Amberlyst ion-exchange resin (approximately 3-4 g per gram of substrate, dried at 110°C under high vacuum) added in one portion. The mixture was stirred for up to 24 hours at room temperature and then 20 filtered. The resin beads were washed thoroughly with fresh benzene and methylene chloride. The filtrates were combined, washed with water, dried over magnesium sulfate, and concentrated in vacuo. The product 11a was purified in 85% yield by column chromatography. Mp 192-194°C.

25 Anal. Calcd. for C₁₅H₁₈O₄: C, 68.69; H, 6.92. Found: C, 68.52; H, 6.99. ¹H NMR (CDCl₃) δ broad roll centered at about 12.0 (1H), 7.85 (1H, d, J = 9 Hz), 6.50 (1H, d, J = 9 Hz), 3.89 (3H, s), 2.94 (2H, s), and 1.69 (10H, broad m) in ppm downfield from TMS. ¹³C NMR (CDCl₃) 164.5, 160.6, 158.7, 132.7, 113.6, 106.2, 104.5, 94.2, 55.7, 38.0, 37.1 (2C's), 24.9, and 23.2 (2C's) in ppm downfield from TMS.

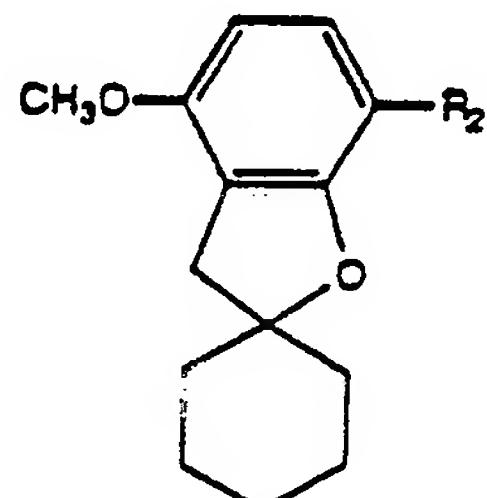
30 FIGURE 1 shows the infrared (KBr) spectrum. IR (KBr) 1660, 1615, 1445, 1435, 1385, and 1100 cm⁻¹. Cyclization 35 was also accomplished by heating the phenol precursor in

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either a 50/50 4N hydrochloric acid/isopropanol solvent mixture or a boron trifluoride etherate solution in THF. This latter cyclization procedure is not recommended for any phenol precursor other than 12a, however.

5

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11a R₂ = -COOH

11b R₂ = -CHO

11c R₂ = -CONHCH₃

11d R₂ = -CONH'BU

11e R₂ = -CH₂OH

11

15

6.5. 7-FORMYL-4-METHOXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (11b)

20

The phenol precursor 12b was treated with Amberlyst resin as described in the previous section. The product 11b was isolated and purified by column chromatography (83% yield). Mp 62-63°C. Anal. Calcd. for C₁₅H₁₈O₃:

C, 73.15; H, 7.37. Found: C, 73.22; H, 7.40. ¹H NMR (CDCl₃) δ 10.16 (1H, s), 7.66 (1H, d, J = 9 Hz), 6.46 (1H, d, J = 9 Hz), 3.88 (3H, s), 2.88 (2H, s), 1.4-2.0 (10H, broad m).

Compound 11b is easily transformed to 11e by a metal hydride reduction step or other means well-known in the art.

6.6. COMPLEMENT INHIBITION

The dihydrobenzofuran and spirobenzo-furancyclohexane compounds of the general formulae 3, 4, the synthetic intermediates of the general formula 5, and the salts thereof of the present invention were assayed for their ability to inhibit complement by the methods described infra.

35

6.6.1. DEMONSTRATION OF INHIBITION OF C3a AND C5a PRODUCTION

The ability to inhibit complement was tested by assaying for specific inhibition of C3a and C5a production. For all experiments, a single human serum pool, to be used as a source of complement, was aliquoted and stored frozen at -70°C. Human IgG was heat-aggregated, aliquoted, and stored frozen at -70°C. For each experiment, serum aliquots were equilibrated at 37°C with varying concentrations of the compounds tested. The classical complement pathway was initiated by the addition of aggregated human IgG. Control samples containing no IgG were always included. After a fixed reaction time of 10 minutes (determined in an earlier time-course study to provide a convenient time interval during which the production of C5a or C3a is nearly complete, i.e., greater than 90%), the levels of the released complement peptides (C5a or C3a) were determined by radioimmunoassay using commercially available radioimmunoassay (RIA) kits (C5a RIA, UpJohn Cat. No. 3250-02; C3a RIA, UpJohn Cat. No. 3245-01; C5a RIA, Amersham Cat. No. RPA.520; C3a RIA, Amersham RPA.518).

Since a competitive immunoassay was used, complement peptide (C5a and C3a) concentrations varied inversely with the counts. The Counts Bound (CB) for a sample was defined as the total counts (in counts per minute, cpm) measured in the pellet minus the counts measured in a non-specific binding (NSB) control. The NSB control was a sample containing only tracer peptide (¹²⁵I-labelled) and second precipitating antiserum; it contained no C5a- or C3a-specific antiserum.

The y-axis in FIGURE 2 represents the fraction inhibition. The fraction inhibition is equal to the Counts Bound (CB) for a "sample," less the CB in the "sample with no added compound," divided by the CB for the "no IgG

control" less the CB in the "sample with no added compound."

$$\text{INHIBITION} = \frac{[(\text{CB sample}) - (\text{CB no compound})]}{[(\text{CB no IgG}) - (\text{CB no compound})]}$$

When C5a production is inhibited at concentrations of added compound at which C3a production is unaffected, the data suggest that complement inhibition is directed toward the C5 activation step. FIGURE 2 is representative of the data plots obtained from the assays described above. The results are summarized in Table VI and demonstrate that the complement inhibition activities exhibited by some of the compounds appear to be directed toward inhibition of C5 activation, and that the inhibitory activities are comparable to that of K-76 COONa itself.

15

TABLE VI

IN VITRO COMPLEMENT INHIBITION IN HUMAN SERUM

	<u>Compound</u>	<u>IC₅₀ (mM)*</u>	
		<u>C3a</u>	<u>C5a</u>
20	(+)-22a	11	6
	(+)-20a	>14 ^b	8
	(-)-20a	11	6
	(+)-23a	>14 ^b	>14 ^b
25	12b	18	9
	12a	13	8
	11a	22	5
	K-76 COONa	- ^c	3

* The concentration of compound required to inhibit C3a or C5a production 50% relative to control samples which contained no test compound.

^b The inhibition observed at this concentration was less than about 50%.

^c Only marginal inhibition was observed at a concentration of about 8.5 mM.

6.6.2. DEMONSTRATION OF INHIBITION OF COMPLEMENT-MEDIATED HEMOLYSIS

The ability to inhibit complement was also tested by assaying for inhibition of complement-mediated red cell lysis (hemolysis). The inhibition of hemolysis was determined as a function of compound concentration. The compounds to be tested were diluted in 0.1 M Hepes buffer (0.15 N NaCl, pH 7.4), and 50 μ l were added to each well of a V-bottom microtiter plate. Human serum, used as the complement source, was diluted 1 to 500 in Hepes buffer, and 50 μ l were added to each well. Next, commercially available sheep erythrocytes with anti-sheep antibody (Diamedix Cat. No. 789-001) were used as received and added at 100 μ l/well to initiate the complement pathway leading to hemolysis. The plate was incubated for 60 minutes at 37°C and then centrifuged at 500 x g for 10 minutes. The supernatants were removed and placed in a flat-bottom microtiter plate. The extent of hemolysis was measured as a function of the sample absorbance at 410 nm. The maximal absorbance (corresponding to maximal hemolysis), A_{max} , was obtained from the absorbance value of an erythrocyte sample containing only human serum, A_s , less the absorbance of a sample containing only the red cells, A_0 . Thus, $A_{max} = A_s - A_0$. The difference between the absorbance of an erythrocyte sample containing both human serum and inhibitive compound, and the absorbance of a cell sample containing inhibitive compound only, was defined as A_{sample} . The inhibition, IH, was expressed as the fraction $(A_{max} - A_{sample})/A_{max}$, and IH_{50} was defined as the concentration of inhibitive compound required to produce a value of $IH = 1/2$.

FIGURE 3 shows the plot of inhibition, IH, versus the concentration of **11a** added to the cell samples. The concentration of **11a** corresponding to IH_{50} is approximately 0.9 mM. Table VII summarizes the results of sever-

al assays using some of the compounds of the present invention and shows their effectiveness in inhibition of hemolysis.

5

TABLE VII
INHIBITION OF COMPLEMENT-MEDIATED HEMOLYSIS

<u>Compound</u>	<u>IHO₅₀ (mM)*</u>	<u>N=</u>
11a	1.33 (± 0.49)	10
10 12a	2.10 (± 0.71)	2
14a	3.0	
15a	0.82 (± 0.16)	2
(+)-20a	0.68 (± 0.03)	2
(-)-20a	0.38 (+0.11)	2
15 (++)-23a	2.1	
(-)-23a	2.4	
30a	0.53 (± 0.19)	23
31a	1.45 (± 0.21)	2
K-76 COONa	0.57 (± 0.17)	9

20

*The concentration of compound required to produce a value for hemolysis inhibition (IH, as defined supra) of 1/2.

25

6.7. IMMUNOSUPPRESSIVE ACTIVITIES

The dihydrobenzofuran and spirobenzofurancyclohexane compounds of the general formulae 3, 4, intermediate 5, and the salts thereof of the present invention 30 were tested for their ability to inhibit cell-mediated immune activity. The specific activities demonstrated by the tested compounds included the inhibition of natural killer (NK) activity, the inhibition of peripheral blood lymphocyte (PBL) proliferation, and the inhibition of cell 35

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surface interleukin-2 receptor expression as described infra. In addition, the ability of compound 11a to inhibit the proliferation of Chinese hamster ovary (CHO) cells was tested; no inhibition of CHO cells was observed, 5 suggesting that the compounds inhibitory activities were specific to the lymphoid cells of the immune system. The experiments were conducted using in vitro assays for determining dose-dependent effects of the compounds of the invention on immune function.

10

6.7.1. DEMONSTRATION OF INHIBITION OF NATURAL KILLER ACTIVITY

Compounds were tested for their effects on the ability of peripheral blood mononuclear cells to lyse NK-sensitive target cells, K562. NK activity was determined 15 using ⁵¹Cr-labeled K562 erythromyeloid leukemia cells as targets, and normal peripheral blood mononuclear cells as effector cells, in a four hour cytotoxicity assay. Effector cells were isolated from fresh blood by Ficoll-Hypaque 20 gradient centrifugation, and 100 µl of a 5 x 10⁵ cell/well suspension were added to each well of a V-bottom microtiter plate. The compound 11a was diluted in RPMI 1640 medium containing 10% fetal calf serum and dispensed at 100 µl per well. Target cells (K562) were labeled for 30 25 minutes with 100 µCi of ⁵¹Cr, washed thoroughly and dispensed in a 20 µl volume at 10⁴ cells/well. These cell concentrations resulted in an effector-to-target cell ratio of 50:1. The microtiter plate was centrifuged at 50 x g for 5 minutes and incubated in a humidified chamber 30 with 5% CO₂ at 37°C. After 4 hours, 100 µl were removed from each well and the radioactivity was measured with a LKB 1275 gamma counter. The percent specific lysis was calculated as follows:

$$35 \% \text{ specific lysis} = [(\text{EXP} - \text{SR}) / (\text{TOTAL} - \text{B})] \times 100$$

where EXP (experimental value) was obtained using effector and target cells; SR (the spontaneous release) was obtained from target cells incubated with media alone; TOTAL release was obtained by hypotonic lysis in water; and B represents instrumental background. Means were calculated from quadruplicate wells and the standard deviations never exceeded 10%. Viability of the effector cells incubated with the test compound was determined by trypan blue exclusion.

The results of the inhibition of natural killer activity for 7-carboxy-4-methoxyspiro[benzofuran-2(3H)-cyclohexane] (11a) sodium salt are summarized in Table VIII.

15

TABLE VIII
INHIBITION OF NATURAL KILLER ACTIVITY BY 7-CARBOXY-4-METHOXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (11a), SODIUM SALT

Concen- tration (mM)	<u>Specific Lysis</u> %	Spont. Release	Viability
		Target Cells Alone (K562)	of Effector Cells ^a
0	17	6	95
0.4	7	5	95
0.8	4	5	95
1.6	1	5	95
3.2	-1	5	76
6.4	18	23	5

^aViability was determined by trypan blue exclusion.

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The data listed in Table VIII indicate that compound 11a can, at appropriate concentrations, effectively inhibit natural killer activity.

5 6.7.2. DEMONSTRATION OF INHIBITION OF PROLIFERATION
 OF PERIPHERAL BLOOD LYMPHOCYTES

The proliferation of human peripheral blood lymphocytes (PBL) in response to phytohemagglutinin (PHA, Wellcome) or anti-CD3 monoclonal antibody (OKT-3, Ortho) was assessed by the incorporation of ^3H -thymidine. Compound 10 11a was diluted in culture media to the desired concentrations, human PBL were added to a final concentration of 10^6 cells/ml, and then either PHA (Wellcome) or anti-CD3 (OKT3, Ortho) antibody (final concentration, 1 mg/ml) was 15 added to initiate proliferation. The final volume per sample was 100 μl . The cells were incubated for 72 hours after stimulation, pulsed with 1 μCi ^3H -thymidine per sample for 4 hours, harvested, and counted in a scintillation counter. Separate experiments, conducted on samples 20 exposed only to varying amounts of 11a without the stimulant, showed that the PBL were viable, as determined visually by trypan blue exclusion, in the concentration ranges of 11a used above. The results (Figs. 4, 5) showed that compound 11a inhibited PBL proliferation in a dose-dependent manner. Half-maximal inhibition was observed at 25 about 0.5 mM of 11a when cultures were stimulated with either PHA (Fig. 4) or anti-CD3 antibody (Fig. 5). Because cell viability was unaffected by the presence of 11a (see supra), the inhibition apparently was not due to 30 cytotoxic effects. Additional compounds of the invention were tested for their immunosuppressive activities, with the results presented in Table IX.

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TABLE IX
INHIBITION OF PROLIFERATION OF
PERIPHERAL BLOOD LYMPHOCYTES

5 <u>Compound</u>	<u>IP₅₀ (mM)*</u>	
	PHA-stimulated PBL	OKT-3-stimulated PBL
11a	0.4	0.5
15a	0.7	0.4
(+)-20a	2.1	1.7
(-)-23a	2.8	2.0
10 K-76 COONa	0.5	0.5

*The concentration of compound required to inhibit PBL proliferation 50% relative to control samples which contained no test compound.

15

The results (Table IX) revealed that each of the tested compounds inhibited PBL proliferation.

Release of cell surface interleukin-2 receptor 20 (IL-2R) or CD8 antigen from lymphocytes is a correlate of T cell activation (Rubin et al. J. Immunol. 1985, 135, 3172- 77; Rubin et al. Fed. proc. 1985 44, 946; Fujimoto, J. et al. J. Exp. Med. 1983, 159, 752-66; Tomkinson, B. et al. 2d Annual Conference on Clinical Immunol. Washington, 25 D.C., October 30, 1987). In some experiments, the level of IL-2R or CD8 protein released into the supernatant of the PBL cultures was assessed by removing aliquots therefrom just prior to pulsing with ³H-thymidine. Commercially available enzyme immunoassay kits (CELLFREE™ IL-2R, Cat. 30 No. CK1020, T Cell Sciences, Inc., Cambridge, MA, or CELL-FREE™ T8/CD8, Cat. No. CK1040, T Cell Sciences) were used to determine the levels of the two analytes. The results using compound 11a showed that 11a was an inhibitor of both IL-2R (Fig. 6) and CD8 (Fig. 7) protein release, in 35 stimulated PBL cultures.

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6.7.3. DEMONSTRATION OF INHIBITION OF CELL SURFACE
INTERLEUKIN-2 RECEPTOR EXPRESSION

The interleukin-2 receptor (IL-2R) is not detectable on the surface of resting T cells. Upon activation by 5 specific antigens or mitogens, T cell proliferation is mediated by an autocrine mechanism whereby activated cells secrete interleukin-2 and express cell surface IL-2R (Meuer, S.C. et al. Proc. Natl. Acad. Sci. U.S.A. 1984, 81, 1509; Tsudo, M., et al. J. Exp. Med. 1984, 160, 612-10 617; Waldmann, T.A., et al. J. Exp. Med. 1984, 160, 1450-1466).

Compounds were tested for their ability to inhibit T cell activation, as indicated by inhibition of cell-surface IL-2R expression. PBL cultures (1.5 ml; 24-well 15 plates) were stimulated with PHA (1 µg/ml) for 72 hours in the absence or presence of varying amounts of compound 11a. Subsequently, the cells were stained using fluorescein isothiocyanate (FITC)-labeled anti-IL-2R antibody (Act-T-Set IL-2R, Cat. No. AA2009, T Cell Sciences, Inc., 20 Cambridge, MA) and analyzed by flow cytometry (Ortho System 30) (Table X).

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TABLE X
INHIBITION OF CELL SURFACE IL-2R EXPRESSION BY
7-CARBOXY-4-METHOXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE]

<u>(11a), SODIUM SALT</u>		
<u>Concentration of 11a (mM)</u>	<u>Number of Cells Positive for IL-2R</u>	<u>Percentage Positive^a</u>
0	4845	96.9
0.44	913	18.3
1.76	225	4.5

Based upon a total cell count of 5000.

The results listed in Table X indicate a dramatic reduction in the number of PBL expressing the IL-2R in the presence of 11a. We also observed that the amount of IL-2R cell surface expression was reduced in the presence of 11a. The data thus demonstrate that compound 11a significantly suppresses IL-2R cell-surface expression, indicating that this compound can effectively inhibit T cell activation in PBL cultures.

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6.7.4. LACK OF INHIBITION OF CHO CELL PROLIFERATION

The specificity of compound 11a's inhibitory activity upon immune cells was demonstrated by assaying for 11a's ability to inhibit the proliferation of Chinese hamster ovary (CHO) cells. Non-confluent CHO cells were allowed to proliferate for 4 hours in a 96-well plate at a volume of 50 μ l per well in the absence or presence of compound 11a at a concentration of 1.8 mM. The cells were pulsed with 0.5 μ Ci of 3 H-thymidine per sample for 4 hours, har-

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vested, and the amount of incorporated ^3H -thymidine was determined by scintillation counting. The CHO cells allowed to proliferate in the absence of added compound yielded a value of 57025 (± 7530) CPM. The value for the 5 sample grown in the presence of 11a was 52549 (± 6272) CPM. These values revealed no statistical difference in the proliferation rate of the two samples. Because the CHO cell line is of the non-lymphoid type, this result suggests that the compounds of the present invention do not generally inhibit mammalian cell proliferation, but inhibit activation and/or proliferation of PBL cultures.

7. EXAMPLES OF 6-CARBOXYL-4-SUBSTITUTED-
SPIRO[BENZOFURAN-2(3H)-CYCLOHEXANES]

- 15 7.1. 6-CARBOXYL-4-METHOXYSPIRO[BENZOFURAN-
2(3H)-CYCLOHEXANE] SODIUM SALT (44a)
AND 6-CARBOXYLATE-4-ETHOXY-SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] SODIUM
SALT (44b)

Preparation of 33a-b. Methyl 3,5-dihydroxybenzoate 20 (10 g, 59.5 mmol) in dry acetone (200 ml) was added to dry K_2CO_3 (8.2 g, 59.4 mmol). Dimethylsulfate (for 33a; 7.5 g, 59.5 mmol) was added to the mixture, which was refluxed for 24 h. The mixture was filtered. Ether (200 ml) was added to the solution and was extracted with H_2O (100 ml x 2). The organic solution was dried over MgSO_4 ; after removal of solvent, a crude product (9.0 g) was purified by chromatography (1:19 EtOAc/ CH_2Cl_2) to afford 33a as a white solid, yield 3.9 g (36%). mp 95-97°C; TLC R_f 0.58 (1:9 EtOAc/ CH_2Cl_2). NMR (CDCl_3) δ 7.26-7.13 (m, 2H), 6.63 (m, 1H), 5.90 (s, 1H, OH), 3.90 (s, 3H), 3.80 (s, 3H). For 33b: The 30 procedure of 33a was modified by using diethyl sulfate as alkylating agent. 33b: mp 98-100°C; yield, 33%; TLC R_f 0.51 (1:9 EtOAc/ CH_2Cl_2). NMR (CDCl_3) δ 7.16-7.14 (m, 2H),

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6.62 (m,1H), 5.70 (s,1H,OH), 4.07-4.00 (q,2H), 3.90 (s,1H), 1.41-1.38 (t,3H).

Preparation of 34a-b. NaH (50% in mineral oil; 2.2g, 91.7 mmol) was washed with hexane (40 ml x 2) under N₂. Dry 5 DMF (50 ml) was added and the mixture was cooled to 0°C. A solution of 33a (7.6 g, 41.7 mmol) in dry DMF (40 ml) was added slowly to the mixture, and then stirred for 1 h at 25°C. The mixture was recooled to 0°C, and a solution of MOMCl (3.7 g, 45.8 mmol) in dry DMF (20 ml) was added 10 dropwise. The mixture was stirred at 25°C for 3 h. Ether (200 ml) was added to the mixture and washed with H₂O (100 ml x 4). The ether portion was dried (MgSO₄) and concentrated to give a pale yellow liquid as 34a; yield 8.2 g (87%). TLC R_f 0.44 (CH₂Cl₂). IR (neat) 2950, 1740, 1600, 15 1460, 1430, 1320, 1240, 1150, 1060, 1020, 770 cm⁻¹. NMR (CDCl₃) Δ7.32-7.31 (m,1H), 7.24-7.23 (m,1H), 6.79 (m,1H), 5.19 (s,2H), 3.90 (s,2H), 3.83 (s,3H), 3.48 (s,3H). For 34b: Procedure for 34a was modified. Yield, 95%; TLC R_f 0.42 (CH₂Cl₂). IR (neat) 2980, 1725, 1600, 1450, 1300, 20 1240, 1150, 1060, 1030, 770 cm⁻¹. NMR (CDCl₃) δ 7.30-7.29 (m,1H), 7.23-7.22 (m,1H), 6.79-6.78 (m,1H), 5.19 (s,2H), 4.09-4.02 (q,2H), 3.90 (s,3H), 3.48 (s,3H), 1.44-1.39 (t,3H).

Preparation of 35a-b. LiAlH₄ (1.7 g, 44.2 mmol) was 25 suspended in dry THF (150 ml) under N₂ and cooled to 0°C. A solution of 34a (10 g, 44.2 mmol) in THF (20 ml) was added dropwise. The mixture was stirred for 3 h at 25°C. Excess LiAlH₄ was carefully decomposed by additional ice. 30 The mixture was filtered through silica gel and rinsed with ether (150 ml). The solution was washed with H₂O (50 ml x 2), dried and concentrated to give a colorless liquid as 35a, yield 6.7g (76%). TLC R_f 0.38 (1:4 EtOAc/CH₂Cl₂). IR (neat) 3400, 2920, 1600, 1460, 1290, 1150, 1050, 925, 35 840cm⁻¹. NMR (CDCl₃) Δ6.67-6.50 (m,3H), 5.16 (s,2H), 4.65

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(s, 2H), s, 3H), 3.50 (s, 3H), 2.56 (s, H, OH). 35b was prepared by the same procedure that used for 35a: yield, 90-92%; TLC R_f 0.52 (1:4 Et₂O/CH₂Cl₂). IR (neat) 3400, 2940, 1600, 1460, 1390, 1280, 1210, 1155, 1140, 1030, 925, 850 cm⁻¹. NMR (CDCl₃) δ 6.80-6.57 (m, 3H), 5.27 (s, 2H), 4.70 (s, 2H), 4.20-3.97 (q, 2H), 3.50 (s, 3H), 2.00 (s, H, OH), 1.50-1.33 (t, 3H).

Preparation of 36a-b. To a solution of 35a (2.2 g, 11.1 mmol) and imidazole (1.51 g, 22.2 mmol) in CH₂Cl₂ (50 ml), was added slowly TBDMSCl (2.0 g, 13.3 mmol) dissolved in CH₂Cl₂ (10 ml). The mixture was stirred at 25°C for 3 h. The mixture was washed with H₂O (100 ml x 2), dried with (MgSO₄) and concentrated to give a colorless liquid, 3.3 g (94%) as 36a. TLC R_f 0.8 (4:6 hexane/CH₂Cl₂). IR (neat) 2940, 1600, 1460, 1365, 1300, 1250, 1210, 1190, 1150, 1100, 1055, 1025, 925, 835, 765 cm⁻¹. NMR (CDCl₃) δ 6.62-6.61 (m, 1H), 6.58-6.56 (m, 1H), 6.49-6.47 (t, 1H), 5.15 (s, 2H), 4.69 (s, 2H), 3.78 (s, 3H), 3.47 (s, 3H), 0.95 (s, 9H), 0.10 (s, 6H). For 36b: It was prepared by the procedure that used for 36a; yield, 90-95%. IR (neat) 2940, 1600, 1460, 1390, 1370, 1290, 1250, 1150, 1100, 1030, 940, 840, 775 cm⁻¹. NMR (CDCl₃) δ 6.61-6.59 (m, 1H), 6.56-6.54 (m, 1H), 6.48-6.47 (t, 1H), 5.15 (s, 2H), 4.67 (s, 2H), 4.04-3.97 (q, 2H), 3.47 (s, 3H), 1.43-1.37 (t, 3H), 0.94 (s, 9H), 0.10 (s, 6H).

Preparation of 38a-b. To a solution of 36a (8.2 g, 26.2 mmol) and TMEDA (4.6 g, 39.4 mmol) in dry THF (200 ml) under N₂ at 0°C, n-BuLi (12 ml, 28.8 mmol) was added slowly. The solution was stirred 30 min at 0°C and then 1.5 h at 25°C. The solution was recooled to -78°C, CuI (7.5 g, 39.4 mmol) was added in one portion under positive N₂ stream. The mixture was stirred for 1.5 h at -78°C to -40°C. The mixture was recooled to -78°C, bromide 37 (5.5 g, 31.5 mmol) in THF (20 ml) was added dropwise, and the

mixture was stirred 4 h from -78°C to 25°C. Ether (200 ml) was added to the solution and washed with 20% NH₄OH solution until the aqueous layer was no longer blue, dried and concentrated to give a brown liquid (10.4 g). Purification by chromatography (1:19 EtOAc/hexane) afforded a colorless liquid as product **38a** (8.7 g, 82%). TLC R_f 0.70 (1:9 EtOAc/hexane). IR (neat) 2930, 1610, 1590, 1460, 1430, 1260, 1200, 1100, 1080, 1030, 840, 780 cm⁻¹. NMR (CDCl₃) δ 6.68 (s, 1H), 6.61 (s, 1H), 5.22-5.18 (m, 1H), 5.13 (s, 2H), 4.71 (s, 2H), 3.79 (s, 3H), 3.44 (s, 3H), 3.26 (s, 2H), 1.97-1.88 (m, 4H), 1.62-1.46 (m, 4H), 0.94 (s, 9H), 0.10 (s, 6H). For **38b**: Preparation was essentially that used for **38a**. Yield, 60-62%. TLC R_f 0.66 (1:9 EtOAc/hexane). IR (neat) 2940, 1610, 1590, 1440, 1390, 1370, 1260, 1200, 1150, 1100, 840, 770 cm⁻¹. NMR (CDCl₃) δ 6.67 (s, 1H), 6.58 (s, 1H), 5.25-5.22 (m, 1H), 5.13 (s, 2H), 4.69 (s, 2H), 4.03-3.96 (q, 2H), 3.44 (s, 3H), 3.28 (s, 2H), 2.00-1.89 (m, 4H), 1.76-1.47 (m, 4H), 1.39-1.35 (t, 3H), 0.94 (s, 9H), 0.10 (s, 6H).

Preparation of **39a-b**. To the solution of **38a** (7.2 g, 14.7 mmol) in THF (100 ml), (Bu)₄ NF (1.0 M in THF, 21 ml, 21 mmol) was added slowly at 25°C. Solution was stirred at 25°C for 2 h. Ether (100 ml) was added to the solution, then washed with 5% HCl (20 ml) and H₂O (100 ml x 2). The organic layer was dried and concentrated to give a colorless liquid as **39a**, yield 6.0 g (90%). TLC R_f 0.53 (2:3 EtOAc/hexane). IR (neat) 3380, 3920, 1610, 1590, 1450, 1430, 1400, 1200, 1160, 1110, 1080, 1030, 960, 925, 830 cm⁻¹. NMR (CDCl₃) δ 6.70 (s, 1H), 6.61 (s, 1H), 5.21-5.18 (m, 1H), 5.14 (s, 2H), 4.62 (s, 2H), 3.79 (s, 3H), 3.44 (s, 3H), 3.26 (s, 2H), 2.00-1.87 (m, 4H), 1.62-1.48 (m, 4H). For **39b**: Preparation was essentially that used for **39a**. Yield, 95%. TLC R_f 0.58 (2:3 EtOAc/hexane). IR (neat) 3400, 2940, 1610, 1590, 1440, 1390, 1200, 1150, 1120,

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1070, 1030, 925, 820 cm⁻¹. NMR (CDCl₃) δ 6.69 (s, 1H), 6.58 (s, 1H), 5.30-5.25 (m, 1H), 5.14 (s, 2H), 4.59 (s, 2H), 4.03-3.96 (q, 2H), 3.44 (s, 3H), 3.28 (s, 2H), 2.00-1.88 (m, 4H), 1.60-1.46 (m, 4H), 1.39-1.35 (t, 3H).

5 **Preparation of 40a-b.** To a mixture of PCC (5.5 g, 25.6 mmol) in CH₂Cl₂ (100 ml) at 25°C was added 39a (4.6 g, 15.7 mmol) in CH₂Cl₂ (50 ml) slowly. The mixture was stirred at 25°C for 4 h more. The mixture was filtered through silica gel and rinsed with EtOAc (40 ml). The solution was washed with H₂O (50 ml), dried (MgSO₄) and concentrated to give a yellow liquid which then was purified by chromatography (3:7 EtOAc/hexane) to afford a pale yellow liquid as 40a, yield 4.2 g (92%). TLC R_f 0.66 (3:7 EtOAc/hexane). IR (neat) 2920, 1690, 1580, 1450, 1430, 1380, 1300, 1200, 1150, 1110, 1070, 1020, 920, 840, 740, 720 cm⁻¹. NMR (CDCl₃) δ 9.89 (s, 1H), 7.23 (s, 1H), 7.10 (s, 1H), 5.22 (s, 2H), 5.20-5.18 (m, 1H), 3.86 (s, 3H), 3.46 (s, 3H), 3.33 (s, 2H), 1.98-1.88 (m, 4H), 1.61-1.48 (m, 4H).
For 40b: Procedure of preparation was essentially that
10 used for 40a. Yield, 75-81%. TLC R_f 0.56 (1:4 EtOAC/hexane). IR (neat) 2920, 1700, 1585, 1440, 1380, 1310, 1200, 1160, 1110, 1070, 1030, 960, 920, 845, 740, 720 cm⁻¹. NMR (CDCl₃) δ 9.88 (s, 1H), 7.22 (s, 1H), 7.08 (s, 1H), 5.25-5.22 (m, 1H), 5.21 (s, 2H), 4.12-4.05 (q, 2H), 3.47 (s, 3H), 3.36 (s, 2H), 2.00-1.89 (m, 4H), 1.60-1.38 (m, 4H), 1.43-1.39 (t, 3H).

15 **Preparation of 41a-b.** To a solution of 40a (4.2 g, 14.5 mmol) in 2-propanol (30 ml) and THF (15 ml) at 0°C was added slowly aq. 4N HCl solution (40 ml, 160 mmol) in 20 2-propanol (10 ml). The solution was then stirred at 25°C for 16 h or until disappearance of 40a (by TLC). The solution was extracted with ether (50 ml x 3). The ether solution was washed with H₂O (30 ml), then dried and concentrated. The crude product was purified by chromatogra-
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phy (3:7 EtOAc/hexane) to afford **41a** as white solid, yield 3 g (84%). mp 153-155°C. TLC R_f 0.60 (3:7 EtOAc/hexane). NMR ($CDCl_3$) δ 9.90 (s, 1H), 7.04 (m, 2H), 5.78 (s, 1H, OH), 5.68-5.65 (m, 2H), 3.90 (s, 3H), 3.48 (s, 2H), 2.09-2.01 5 (m, 4H), 1.96-1.90 (m, 4H). For **41b**: Procedure of preparation was essentially that used for **41a**. Yield, 76%; TLC R_f 0.34 (1:4 EtOAc/hexane). NMR ($CDCl_3$) δ 9.86 (s, 1H), 7.00 (m, 2H), 5.80 (s, 1H, OH), 5.70-5.66 (m, 2H), 4.13-4.06 (q, 2H), 3.47 (s, 2H), 2.05-2.02 (m, 2H), 1.92-1.88 (m, 2H), 10 1.63-1.54 (m, 4H), 1.46-1.41 (t, 3H).

Preparation of 42a-b. Amberlyst 15 (10 g) was added in one portion to the solution of **41a** (3.3 g, 13.4 mmol) in CH_2Cl_2 (100 ml). The mixture was stirred at 25°C for 6 h. The mixture was filtered and the solution was washed 15 with H_2O (100 ml), dried and concentrated to give a crude product (3 g) which then was purified by chromatography (1:4 EtOAc/hexane) to afford **42a** as a white solid (2.5 g, 76%). TLC R_f 0.57 (1:4 EtOAc/hexane). IR (KBr) 2930, 1690, 1600, 1430, 1390, 1350, 1325, 1220, 1120, 1030, 920, 830, 20 805, 745 cm^{-1} . NMR ($CDCl_3$) δ 9.85 (s, 1H), 6.93 (s, 1H), 6.89 (s, 1H), 3.88 (s, 3H), 2.94 (s, 2H), 1.86-1.64 (m, 6H), 1.55-1.45 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 191.77, 160.53, 156.90, 138.19, 121.25, 105.65, 102.81, 90.36, 55.58, 38.48, 37.23, 25.05, 22.95. For **42b**: Procedure of preparation was 25 essentially that used for **42a**. mp 105-106°C. Yield, 82%; TLC R_f 0.55 (1:4 EtOAc/hexane). NMR ($CDCl_3$) δ 9.84 (s, 1H), 6.91 (s, 1H), 6.87 (s, 1H), 4.15-4.08 (q, 2H), 2.95 (s, 2H), 1.86-1.68 (m, 6H), 1.53-1.45 (m, 4H), 1.45-1.40 (t, 3H).

Preparation of 43a-b. To a mixture of aq. 2N NaOH 30 (30 ml) containing Ag_2O (2.4 g, 10.2 mmol) at 50°C, a solution of **42a** (1 g, 4.1 mmol) in EtOH (1 ml) and THF (5 ml) was added slowly. The mixture was stirred for 6 h at 50°C. The mixture was filtered and the aq. solution was washed 35 with ether (50 ml). The aq. solution then was cooled to

0°C, and acidified with conc. HCl solution. The white precipitate that resulted was extracted with ether, dried and concentrated to afford a white solid as **43a**, yield 0.75g (71%). TLC R_f 0.62 (1:9:10 MeOH/CH₂Cl₂/EtOAc). NMR 5 (CDCl₃) δ 7.16 (s, 2H), 3.88 (s, 3H), 2.94 (s, 2H), 1.87-1.65 (m, 6H), 1.55-1.47 (m, 4H). For **43b**: Procedure for preparation was essentially that used for **43a**. mp 190-192°C. TLC R_f 0.62 (1:9:10 MeOH/CH₂Cl₂/EtOAc); yield, 69%. IR (KBr) 3300-2400, 1675, 1595, 1430, 1350, 1325, 1265, 1210, 1120, 10 1100, 1030, 955, 860, 770, 740 cm⁻¹. NMR (CDCl₃) δ 7.14 (s, 2H), 4.15-4.08 (q, 2H), 2.95 (s, 2H), 1.86-1.65 (m, 6H), 1.60-1.45 (m, 4H), 1.45-1.40 (t, 3H). ¹³C NMR (CDCl₃) δ 172.17, 160.08, 155.61, 130.19, 120.54, 105.39, 104.77, 90.04, 63.80, 38.49, 37.24, 25.09, 22.99, 14.84. Anal. Calcd for C₁₆H₂₀O₄: C, 69.54; H, 7.30. Found: C, 69.40; H, 7.35.

Preparation of 44a-b. Sodium hydride (50% in mineral oil, 0.58 g, 24.2 mmol) was washed with hexane (30 ml) under N₂, then dry ether (50 ml) was added. A solution of 20 **43a** (3.2 g, 12.2 mmol) in ether (150 ml) was added to above mixture slowly at 25°C. The mixture was stirred at 25°C for 6 h and a white precipitate resulted. The mixture was extracted with H₂O (50 ml). The aqueous solution was collected and freeze dried to give a white solid (3.2 g, 25 92%). NMR (D₂O) δ 7.10 (s, 1H), 6.92 (s, 1H), 3.85 (s, 3H), 2.82 (s, 2H), 1.70-1.54 (m, 6H), 1.44-1.33 (m, 4H).

For **44b**: Procedure for preparation was essentially that used for **44a**. Yield, 75%. NMR (D₂O) δ 7.09 (s, 1H), 6.90 (s, 1H), 4.19-4.12 (q, 2H), 2.89 (s, 2H), 1.77-1.60 (m, 6H), 1.50-1.40 (m, 4H), 1.40-1.36 (t, 3H).

7.2. 6-FORMYL-4-HYDROXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (52)

Preparation of 45. The procedure for the preparation 35 of **45** was essentially that used for **34** except that two

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molar equivalent of NaH and MOMCl were used. Yield was 83%; TLC R_f 0.65 (1:1 ether/hexane).

Preparation of 46. The procedure for preparation of 46 was essentially that used for 35. Yield was 89%; TLC R_f 0.27 (1:1 ether/hexane).

Preparation of 47. The procedure for preparation was essentially that used for 36. 47: colorless liquid; yield, 83-98%; TLC R_f 0.80 (1:1 ether/hexane). IR (neat) 2960, 1600, 1470, 1400, 1370, 1290, 1260, 1140, 1080, 1040, 930, 10840, 780 cm^{-1} . NMR (CDCl_3) δ 6.92-6.72 (m, 3H), 5.26 (s, 4H), 4.80 (s, 2H), 3.57 (s, 6H), 1.00 (s, 9H), 0.13 (s, 6H).

Preparation of 48. Procedure for preparation of 48 was essentially that used for 38. Crude product was purified by chromatography (1:19 EtOAc/hexane). 48: a clear liquid; yield, 63-79%; TLC R_f 0.46 (1:19 EtOAc/hexane). IR (neat) 2940, 1615, 1590, 1455, 1440, 1400, 1370, 1260, 1160, 1100, 1050, 930, 840, 780 cm^{-1} . NMR (CDCl_3) δ 6.76 (s, 2H), 5.25-5.22 (m, 1H), 5.15 (s, 4H), 4.70 (s, 2H), 3.45 (s, 6H), 3.30 (s, 2H), 2.10-1.96 (m, 4H), 1.95-1.88 (m, 4H), 0.95 (s, 9H), 0.11 (s, 6H).

Preparation of 49. Procedure for preparation 49 was essentially that used for 39. Yield, 95-100%; TLC R_f 0.45 (3:7 EtOAc/ CH_2Cl_2). IR (neat) 3400, 2930, 1620, 1590, 1440, 1290, 1190, 1155, 1110, 1040, 950, 920, 840 cm^{-1} . NMR ²⁵ (CDCl_3) δ 6.78 (s, 2H), 5.24-5.20 (m, 1H), 5.17 (s, 4H), 4.63 (s, 2H), 3.45 (s, 6H), 3.30 (s, 2H), 2.10-1.88 (m, 4H), 1.73 (s, 1H, OH), 1.63-1.48 (m, 4H).

Preparation of 50. Procedure for preparation 50 was essentially that used for 40. Crude product was purified ³⁰ by chromatography (3:7 ether/hexane). Yield, 70-76%; TLC R_f 0.43 (3:7 ether/hexane). IR (neat) 2930, 1700, 1585, 1435, 1380, 1290, 1155, 1105, 1050, 950, 925 cm^{-1} . NMR (CDCl_3) δ 9.89 (s, 1H), 7.29 (s, 2H), 5.23-5.18 (m, 5H), 3.46 (s, 6H), 3.37 (s, 2H), 2.00-1.95 (m, 4H), 1.94-1.87 (m, 4H).

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¹³C NMR (CDCl₃) δ 191.65, 156.49, 135.74, 135.54, 126.38, 121.11, 108.79, 94.39, 51.15, 31.61, 28.95, 25.30, 23.10, 22.46.

Preparation of 51. Procedure for preparation 51 was essentially that used for 41. Crude product was purified by chromatography (3:7 EtOAc/hexane). It should be mentioned that a large amount of expected product decomposed or was trapped on silica gel to give a low yield (28%).

For 51: mp 130-132°C. TLC R_f 0.41 (3:7 EtOAc/hexane). IR 10 (KBr) 3460, 3420, 2940, 1690, 1670, 1595, 1450, 1410, 1340, 1200, 1170, 1140, 1080, 1050, 1035, 1010, 840, 790, 740 cm⁻¹. NMR (CDCl₃) δ 9.83 (s, 1H), 6.96 (s, 2H), 5.72-5.68 (m, 1H), 5.67 (s, 2H, OH), 3.47 (s, 2H), 2.08-2.01 (m, 2H), 2.00-1.92 (m, 2H), 1.67-1.53 (m, 4H).

Preparation of 52. Procedure of preparation 52 was essentially that used for 42. Crude product was purified by chromatography with 3:7 (EtOAc/hexane) as eluent. For 52: TLC R_f 0.53 (3:7 EtOAc/hexane). IR (KBr) 3260, 2940, 1680, 1590, 1460, 1400, 1310, 1280, 1250, 1210, 1175, 1110, 1060, 1000, 930, 920, 840, 800, 740 cm⁻¹. NMR (CDCl₃) δ 9.81 (s, 1H), 6.88 (s, 1H), 6.86 (s, 1H), 2.97 (s, 2H), 1.89-1.65 (m, 6H), 1.58-1.45 (m, 4H).

7.3. 4-BENZYLOXY-6-FORMYL-SPIRO [BENZOFURAN-2(3H)-CYCLOHEXANE] (53a)

Preparation of 53a. Sodium hydride (50% in mineral oil, 62 mg, 2.58 mmol) was washed with hexane (20 ml) under N₂. Dry DMF (5 ml) was added and a solution of 52 (0.30 g, 1.29 mmol) in DMF (10 ml) was added slowly at 25°C. After stirring for 30 min, a solution of benzyl bromide (0.22 g, 1.29 mmol) in DMF (5 ml) was added dropwise. The mixture was warmed to 50°C and stirred for 2 h more. Ether (50 ml) was added to the mixture which was then washed with H₂O (50 ml x 3), dried and concentrated to afford a crude product (0.4 g). Purification by chromatography (1:4 Et₂O/hexane)

gave a pale yellow liquid as **53a**, yield 0.3 g (71%). TLC R_f 0.45 (1:4 Et₂O/hexane). IR (neat) 2940, 2870, 1695, 1600, 1440, 1390, 1355, 1330, 1220, 1210, 1150, 1100, 1045, 920, 830, 805, 750, 700 cm⁻¹. NMR (CDCl₃) δ 9.85 (s, 1H), 5.746-7.34 (m, 5H), 7.01 (s, 1H), 6.91 (s, 1H), 5.14 (s, 2H), 2.98 (s, 2H), 1.87-1.64 (m, 6H), 1.56-1.42 (m, 4H).

**7.4. 4-N-BUTYLOXY-6-FORMYL-[NBU-]SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (53b)**

Preparation of 53b. Sodium hydride (50% in mineral oil, 62 mg, 2.58 mmol) was washed with hexane (30 ml) under N₂, then dry DMF (5 ml) was added. To the mixture, a solution of **52** (0.3 g, 1.29 mmol) in dry DMF (10 ml) was added dropwise. The mixture was stirred for 30 min. A solution of n-butyl p-toluenesulfonate (0.29 g, 1.29 mmol) in DMF (5 ml) was added dropwise. The mixture was warmed to 50°C and stirred for 2 h. Ether (50 ml) was added to the mixture which was washed with H₂O (50 ml x 2), dried and concentrated to give a crude product. Purification by chromatography (1:4 ether/hexane) afforded a pale yellow liquid as **53b**, yield 0.27g (73%). TLC R_f 0.58 (ether/hexane). IR (neat) 2940, 2860, 1690, 1600, 1430, 1380, 1320, 1210, 1100, 1030, 920, 825, 800, 745 cm⁻¹. NMR (CDCl₃) δ 9.84 (s, 2H), 6.92 (s, 1H), 6.87 (s, 1H), 4.07-4.03 (t, 2H), 2.94 (s, 2H), 1.87-1.64 (m, 8H), 1.58-1.43 (m, 6H), 1.01-0.96 (t, 3H).

**7.5. 6-FORMYL-4-PHOXYSPIRO[BENZOFURAN-
2(3H)-CYCLOHEXANE] (53c)**

Preparation of 53c. Under N₂ condition, a solution of **52** (0.4 g, 1.72 mmol) in CH₂Cl₂ was added to a mixture of triphenylbismuth diacetate (1.16 g, 2.08 mmol) and copper powder (11 mg, 0.17 mmol) in CH₂Cl₂ (50 ml). The mixture was stirred at 25°C for 24 h, then filtered through silica gel and rinsed with 10% EtOAc in CH₂Cl₂ solution (20 ml).

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The solution was washed with H₂O (50 ml), dried and concentrated to give the crude product (0.65 g), which then was purified by chromatography (1:4 ether/hexane) to afford a colorless liquid as 53c; yield 0.4 g (75%). TLC R_f 0.59
5 (1:4 ether/hexane). IR (neat) 3060, 2940, 2860, 1695,
1580, 1485, 1430, 1310, 1215, 1050, 1020, 850, 800, 755,
690 cm⁻¹. NMR (CDCl₃) δ 7.40-6.89 (m, 7H), 2.93 (s, 2H),
1.90-1.63 (m, 6H), 1.55-1.42 (m, 4H).

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7.6. 6-FORMYL-4-(2'-HYDROXYETHYLOXY)
SPIRO[BENZOFURAN-2(3H)-
CYCLOHEXANE] (53d)

Preparation of 53d. Sodium hydride (50% in mineral oil, 0.10 g, 2.15 mmol) was washed with hexane (50 ml) under N₂. Dry DMF (15 ml) was added and a solution of 52
15 (0.50 g, 1.29 mmol) in DMF (10 ml) was added slowly at 25°C. After stirring for 30 min, a solution of 2-[*(tert*butyldimethylsilyl)oxy]ethyl bromide in DMF (5 ml) was added dropwise. The mixture was warmed to 50°C and stirred for 3 h more. Ether (50 ml) was added to the
20 mixture, which was then washed with water (50 ml × 3). The ether layer was collected, dried and concentrated to give a crude product (0.9 g). Purification by chromatography (1.5:8.5 ether/hexane) gave a pale yellow solid
0.70g (83%). TLC R_f 0.50 (1:4 ether/hexane). mp 74-75°C.
25 IR (KBR) 2940, 2860, 1695, 1600, 1440, 1390, 1330, 1220,
1140, 1110, 1095, 960, 830, 780 cm⁻¹. NMR (CDCl₃) δ 9.84
(s, 1H), 6.93 (s, 1H), 6.89 (s, 1H), 4.13 (t, 2H), 3.98
(t, 2H), 2.95 (s, 2H), 1.87-1.45 (m, 10H), 0.91 (s, 9H),
30 0.10 (s, 6H).

30 The above product (0.31 g, 0.79 mmol) was dissolved in THF (5 ml), then tetrabutylammonium fluoride (1 ml, 1.0 mmol, 1.0 M in THF solution) was added, solution was stirred at 25°C for 2 h. Ether (30 ml) was added, and the
35 solution was washed with water (50 ml). The ether layer

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was collected, dried and concentrated to afford a pale yellow liquid. Purification by chromatography (1:4 hexane/ether) gave a pale yellow liquid as 53d. Yield, 0.17g (77%). TLC R_f 0.34 (1:4 hexane/ether). IR (neat) 53450(b), 2940, 2870, 1690, 1600, 1440, 1390, 1325, 1220, 1130-1070(b), 1035, 925, 830, 810, 755 cm^{-1} . NMR (CDCl_3) δ 89.84 (s, 1H), 6.93 (s, 1H), 6.91 (s, 1H), 4.18 (t, 2H), 4.00 (t, 2H), 2.97 (s, 2H), 1.87-1.64 (m, 6H), 1.49 (s, b, 4H).

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7.7. 6-FORMYL-4-P-NITROPHENOXYSPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (53e)

Preparation of 53e. Sodium hydride (50% in mineral oil, 0.10 g, 4.58 mmol) was washed with hexane (20 ml) under nitrogen, and dry DMF (50 ml) was added. To the mixture, a solution of 52 (0.50 g, 2.15 mmol) in DMF (15 ml) was added slowly at 25°C. The mixture was stirred for 30 min. A solution of 1-fluoro-4-nitrobenzene in DMF (10 ml) was added slowly. The mixture was then warmed to 35°C and stirred for 3 h. Ether (100 ml) was added to the mixture after cooling to 25°C which was washed with water (50 ml x 3). Ether layer was collected, dried and concentrated to give a crude product (0.79 g). Purification by chromatography (1:9 EtOAc/hexane) afforded a yellow sticky oil as expected product 53e. Yield, 0.41g (54%). TLC R_f 25 0.32 (1:4 EtOAc/hexane). IR (neat) 2940, 2860, 1695, 1570, 1515, 1490, 1430, 1345, 1310, 1240, 1165, 1110, 1065, 1050, 1025, 920, 865, 850, 800, 750, 745 cm^{-1} . NMR (CDCl_3) δ 9.86 (s, 1H), 8.26-8.23 (d, 1H), 7.06 (s, 1H), 7.06-7.03 (m, 3H), 2.86 (s, 2H), 1.90-1.40 (m, 10H).

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7.8. 6-FORMYL-4-P-FORMYLPHENOXYSPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (53f)

Preparation of 53f. Sodium hydride (50% in mineral oil, 103 mg, 4.31 mmol) was washed with hexane (20 ml) under N_2 . Dry DMF (30 ml) was added and a solution of 52

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(0.40 g, 1.72 mmol) in DMF (5 ml) was added slowly at 25°C, and a solution of p-fluorobenzaldehyde in DMF (5 ml) was added. The mixture was warmed to 50°C and stirred for 24 h. After cooling to 25°C, ether (100 ml) was added, the 5 solution was washed with water (30 ml x 3). The ether layer was collected, dried and concentrated to give a crude product. Purification by chromatography (1:9 EtOAc/hexane) gave a pale yellow solid as expected product 53f. Yield, 0.31 g (53%). 53f: mp 127-129°C. TLC R_f 0.53 10 (1:4 EtOAc/hexane). IR (KBR) 2940, 2840, 1680, 1560, 1500, 1440, 1390, 1320, 1220, 1170, 1050, 840, 800cm⁻¹. NMR (CDCl₃) δ 9.95 (s, 1H), 9.85 (s, 1H), 7.90-7.87 (d, 2H), 7.12 (s, 1H), 7.10-7.07 (d, 2H), 7.04 (s, 1H), 2.87 (s, 2H), 1.90-1.40 (m, 10H).

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7.9. 6-FORMYLSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (53g)

Preparation of 53g. Pyridine (0.34 g, 4.31 mmol) and 52 (0.50 g, 2.15 mmol) were dissolved in methylene 20 chloride (50 ml) and cooled at 0°C under N₂. Triflic (trifluoromethanesulfonic) anhydride (0.40 ml, 2.37 mmol) was introduced dropwise by syringe. The solution was stirred for 1 h at 0°C. The solution was then washed with 5% aq. HCl (10 ml) and water (30 ml x 3), dried and 25 concentrated to give a crude product, which was purified by chromatography (1:9 EtOAc/hexane) to afford the expected product phenol triflate 52. Yield, 0.70 g (90%). Phenol triflate 52: TLC R_f 0.74 (1:4 EtOAc/hexane). IR (neat) 2940, 2860, 1710, 1625, 1590, 1430, 1300, 1250-1200(b), 30 1140, 1010, 980, 920, 850, 830, 800, 770 cm⁻¹. NMR ((CDCl₃) δ 9.89 (s, 1H), 7.25 (s, 2H), 3.12 (s, 2H), 1.92-1.44 (m, - 10H).

Phenol triflate 52 (1.00 g, 2.74 mmol), triphenylphosphine (58 mg, 0.22 mmol), palladium acetate 35

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(25 mg, 0.11 mmol) and triethylamine (1.66 g, 16.4 mmol) were dissolved in DMF (10 ml) and 96% formic acid (0.50 g, 10.8 mmol) in DMF (2 ml) was added. The mixture was stirred at 60°C for 2 h under N₂. The mixture was diluted with water (50 ml) and extracted with ether (50 ml x 3). The ether layer was collected, dried and concentrated to give a brown residue. The crude product was purified by chromatography (1:9 EtOAc/hexane) to give the expected product **53g** as a pale yellow oil. Yield, 0.29 g (49%).

53g: TLC R_f 0.69 (1:9 EtOAc/hexane). IR (neat) 2940, 2860, 1690, 1585, 1490, 1440, 1340, 1310, 1270, 1250, 1150, 1030, 950, 920, 870, 800, 785 cm⁻¹. NMR (CDCl₃) δ 9.90 (s, 1H), 7.36-7.33 (d, 1H), 7.28-7.26 (d, 1H), 7.22 (s, 1H), 3.02 (s, 1H), 1.89-1.43 (m, 10H).

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7.10. 4-BENZYLOXY-6-CARBOXYL SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54a**);
4-N-BUTYLOXY-6-CARBOXYL SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54b**);
6-CARBOXYL-4-PHENOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54c**);
20 6-CARBOXYL-4-(2'-HYDROXYETHYL)OXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54d**);
AND 6-CARBOXYL-4-P-NITROPHENOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54e**)

Preparation of 54a-e. Procedure for preparation of these compounds was essentially that used for **43**. For **54a**:
25 mp 173-175°C; yield, 74-83%; TLC R_f 0.56 (1:4 hexane/ether). IR (KBr) 3100-2500, 1680, 1600, 1420, 1320, 1215, 1095, 950, 735 cm⁻¹. NMR (CDCl₃) δ 7.46-7.34 (m, 5H), 7.26 (s, 1H), 7.18 (s, 1H), 5.13 (s, 2H), 2.97 (s, 2H), 1.86-1.65 (m, 6H), 1.48 (s, br, 4H). Anal. Calcd for C₂₁H₂₂O₄: C, 74.53; H, 6.55. Found: C, 74.44; H, 6.57.

For **54b**: mp 145-147°C; yield, 81%; TLC R_f 0.62 (1:4 hexane/ether). IR (KBr) 3100-2500, 1670, 1600, 1425, 1320, 1275, 1210, 1100, 1030, 940, 865, 765, 745 cm⁻¹. NMR (CDCl₃) δ 7.14 (s, 2H), 4.06-4.02 (t, 2H), 2.94 (s, 2H),

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1.86-1.65 (m, 8H), 1.56-1.44 (m, 6H), 1.01-0.97 (t, 3H).
Anal. Calcd for C₁₈H₂₄O₄: C, 71.01; H, 7.95. Found: C, 71.01;
H, 7.97.

For 54c: mp 173-175°C; yield, 71%; TLC R_f 0.69 (1:4
5 hexane/ether). IR (KBr) 3100-2500, 1680, 1580, 1480, 1420,
1310, 1205, 1050, 950, 865, 765, 685cm⁻¹. NMR (CDCl₃) δ
7.37-6.97 (m, 7H), 2.90 (s, 2H), 1.90-1.60 (m, 6H), 1.50-1.40
(m, 4H). ¹³C NMR (CDCl₃) δ 171.45, 160.88, 156.32, 153.39,
130.62, 129.86, 123.74, 123.56, 118.44, 112.37, 106.59,
10 90.34, 38.70, 37.11, 25.02, 22.90. Anal. Calcd for C₂₀H₂₀O₄:
C, 74.05; H, 6.22. Found: C, 73.97; H, 6.25.

For 54d: Reaction was stirred for 8 h at 50°C. 54d: mp
163-164°C; TLC R_f 0.72 (1:19 MeOH/ether); yield, 94%. IR
(KBr) 3600-3300(b), 2940, 2860, 1690, 1600, 1430, 1325,
15 1320, 1115, 1070, 960, 755, 725 cm⁻¹. NMR (CDCl₃) δ 57.15
(s, 1H), 7.14 (s, 1H), 4.18 (t, 2H), 3.99 (t, 2H), 2.96 (s, 2H)
1.87-1.65 (m, 6H), 1.49 (s, b, 4H). ¹³C NMR (CDCl₃) δ 171.24,
160.26, 155.23, 130.28, 120.61, 105.44, 105.36, 90.21,
69.46, 61.43, 38.48, 37.23, 25.05, 22.96. Anal. Calcd for
20 C₁₆H₂₀O₅: C, 65.74; H, 6.90. Found: C, 65.58; H, 6.96.

For 54e: Mixture was stirred for 24 h at 50°C. Yield,
56%. 54e: mp 190-191°C; TLC R_f 0.80 (ether). IR (KBr)
3100-2500(b), 1690, 1585, 1525, 1490, 1425, 1390, 1310,
1230, 1170, 1115, 1070, 1025, 960, 860, 850, 770, 755 cm⁻¹
25 NMR ((CDCl₃) δ 8.25-8.22 (d, 2H), 7.36 (s, 1H), 7.29 (s, 1H),
7.04-7.01 (d, 2H), 2.84 (s, 2H), 1.90-1.40 (m, 10H). ¹³C NMR
(CDCl₃) δ 170.45, 161.92, 161.35, 150.96, 143.03, 131.22,
126.12, 124.98, 116.93, 113.98, 108.45, 90.76, 38.66,
30 37.05, 24.91, 22.81. Anal. Calcd. for C₂₀H₁₉NO₆: C, 65.03; H,
5.18; N, 3.79. Found: C, 64.96; H, 5.23; N, 3.71.

7.11. 6-CARBOXYLSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54g**) ; AND
4-P-AMINOPHOXY-6-CARBOXYLSPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54h**) ;
6-CARBOXYL-4-P-CARBOXYLPHENOXYSPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**54i**)

5 **54g:** Mixture was stirred at 60°C for 4 h. Yield, 84%.

mp 154-155°C. TLC R_f 0.82 (ether). IR (KBr) 3200-2500(b),
1680(b), 1590, 1440, 1390(b), 1090, 1030, 950, 780,
 770cm^{-1} . NMR (CDCl_3) δ 7.62-7.60 (d,1H), 7.44 (s,1H),
7.21-7.19 (d,1H), 3.01 (s,1H), 1.89-1.43 (m,10H). Anal.

¹⁰ Calcd for $\text{C}_{14}\text{H}_{16}\text{O}_3$: C, 72.39; H, 6.94. Found: C, 72.31; H,
6.95.

¹⁵ **54h:** **54e** (30 mg, 0.81 mmol) dissolved in MeOH (10 ml)
and 10% Pd-C (20 mg) was added. H_2 gas was bubbled through
the mixture for 5 h at 25°C. The mixture was filtered and
MeOH was removed to give a brown oil. The crude product
was purified by preparative TLC plate (1:19 MeOH/ CH_2Cl_2) to
afford a pale yellow solid as product **54h**. Yield, 10 mg
(36%). **54h:** mp 193-195°C. TLC R_f 0.49 (1:9 MeOH/ CH_2Cl_2). IR
(KBr) 3380, 3320, 2940. 2860, 1700, 1600, 1510, 1420,
²⁰ 1320, 1290, 1270, 1210, 1200, 1050, 950, 920, 770cm^{-1} . NMR
(CDCl_3) δ 7.15 (s,1H), 7.00 (s,1H), 6.87-6.84 (d,2H),
6.87-6.84 (d,2H), 2.94 (s,2H), 1.88-1.43 (m,10H). MS m/z
(relative intensity) 339(100), 322(2), 296(5), 282(7),
²⁵ 258(29), 217(61), 196(11), 167(15), 156(7), 130(9),
108(29), 93(95).

³⁰ **54i.** Procedure for preparation of these compounds
was essentially that used for **43**. Mixture was stirred for
24 h at 60°C. The crude product was treated with CH_2N_2
which then was purified by chromatography (1:4 EtOAc/hex-
ane) to give the dimethyl ester of **54i**. Yield, 0.18g
(51%). Dimethyl ester of **54i**: IR (neat) 2940, 2860, 1750,
1590, 1500, 1430, 1350, 1310, 1280, 1250, 1220, 1160,
1110, 1080, 1070, 1050, 1030, 1000, 920, 880, 850, 770,
³⁵ 690 cm^{-1} . NMR (CDCl_3) δ 8.03-8.00 (d,2H), 7.26 (s,1H), 7.21

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(s,1H), 6.98-6.95 (d,2H), 3.91 (s,3H), 3.86 (s,3H), 2.82 (s,2H), 1.88-1.39 (m,10H).

The dimethyl ester of **54i** (0.15 g, 0.38 mmol) was dissolved in THF (10 ml), then a solution of 2N NaOH (5 ml) was added. The solution was warmed to 50°C and stirred for 3 h. After cooling to 25°C, the aq. solution was rinsed with ether (20 ml) and the aq. solution was acidified with conc. HCl. A white precipitate resulted, which was extracted with ether (50 ml x 2). After the precipitate was dried and concentrated, a white solid was resulted as product **54i**. Yield, 0.13 g (93%). **54i** mp 278-280°C. TLC R_f 0.29 (1:9 MeOH/CH₂Cl₂). IR (KBr) 3200-2400(b), 1690, 1605, 1590, 1505, 1425, 1315, 1280, 1220, 1170, 1050, 950, 915, 880, 850, 770, 740, 720cm⁻¹. NMR (MeOH-d₄) δ 8.05-8.02 (d,2H), 7.18 (s,1H), 7.15 (s,1H), 7.03-7.00 (d,2H), 2.84 (s,2H), 1.86-1.43 (m,10H). ¹³C NMR (MeOH-d₄) δ 169.21, 168.97, 162.48, 162.13, 153.28, 133.91, 133.13, 126.71, 124.86, 118.09, 114.23, 108.04, 91.47, 39.76, 37.98, 26.09, 23.90. Anal. Calcd for C₂₁H₂₀O₆: C, 68.47; H, 5.47. Found: C, 68.22; H, 5.48.

- 7.12. SODIUM SALTS OF 4-BENZYLOXY-6-CARBOXYL SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55a**);
4-N-BUTYLOXY-6-CARBOXYL SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55b**);
25 6-CARBOXYL-4-PHENOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55c**);
6-CARBOXYL-4-(2'-HYDROXYETHYLOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55d**);
6-CARBOXYL-4-P-NITROPHENOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55e**);
30 6-CARBOXYL SPIRO[BENZOFURAN-2(3H)-
CYCLOHEXANE] (**55g**); AND
6-CARBOXYL-4-P-CARBOXYLPHENOXY SPIRO
[BENZOFURAN-2(3H)-CYCLOHEXANE] (**55i**)

Preparation of **55a-e,g,i**. Procedure for preparation of these compounds was essentially that used for **44**. NMR spectra for **55a-g** are listed as below. **55a**: NMR (D₂O) δ 7.21-6.88 (s,7H), 4.85 (s,2H), 2.56 (s,2H), 1.43-1.06

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(m, 1OH). **55b:** NMR (D₂O) δ 7.10 (s, 1H), 6.90 (s, 1H), 4.13 (t, 2H), 2.93 (s, 2H), 1.79-1.67 (m, 8H), 1.50-1.41 (m, 6H), 0.93 (t, 3H). **55c:** NMR (D₂O) δ 7.20-6.80 (m, 7H), 2.61 (s, 2H), 1.60-1.12 (m, 10H). **55d:** NMR (D₂O) δ 7.07 (s, 1H), 5.6.92 (s, 1H), 4.18 (t, 2H), 3.93 (t, 2H), 2.96 (s, 2H), 1.68 (s, b, 6H), 1.44 (s, b, 4H). **55e:** NMR (D₂O) δ 8.10-8.07 (d, 2H), 7.13 (s, 1H), 7.11 (s, 1H), 6.98-6.95 (d, 2H), 2.68 (s, 2H), 1.70-1.23 (m, 10H). **55i:** NMR (D₂O) 7.90-7.87 (d, 2H), 7.14 (s, 1H), 7.12 (s, 1H), 7.00-6.97 (d, 2H), 2.76 (s, 2H), 10.1.70-1.27 (m, 10H). **55g:** NMR (D₂O) δ 7.45-7.42 (d, 1H), 7.23-7.20 (m, 2H), 2.93 (s, 2H), 1.65 (s, b, 6H), 1.41 (s, b, 4H). ¹³C NMR (D₂O) δ 177.63, 160.05, 139.25, 133.68, 127.83, 124.60, 112.09, 93.64, 42.45, 38.96, 27.15, 25.37.

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8. EXAMPLES OF 6,7-DISUBSTITUTED
-4-METHOXYSPIRO[BENZOFURAN-
2(3H)-CYCLOHEXANES]

The melting points were measured with a Thomas Hoover apparatus, and are uncorrected; the infrared spectra were obtained with the aid of a Perkin Elmer 281B spectrophotometer, the ¹H NMR spectra were recorded in deuterio-chloroform, unless another solvent is specified, either at 20 90 MHz, in a Varian EM 390 apparatus, or at 300 MHz in a Varian VXR 300 spectrometer. The ¹³C NMR were recorded at 25 75.4 MHz in a Varian VXR 300 spectrometer. All the signals are reported in ppm, downfield from tetramethyl-silane, used as internal reference. The multiplicities of 30 the signals are abbreviated as: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, b=broad signal. The low resolution mass spectra were obtained from a Finnigan 3221-F200 spectrograph, at 70 eV. The elemental analyses were done by Atlantic Mirolab, Inc. (Norcross, GA). The HRMS was obtained at Massachusetts Institute of Technology.

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All the solvents and chemicals used were of good quality. In several cases, some of them were purified and/or dried following preestablished procedures. All the reactions were carried out under a dry nitrogen atmosphere. The column chromatographies were done using MN Silica gel 60, under atmospheric pressure, using different solvent systems (hexane-ether, hexane-ethyl acetate), in which increasing quantities of the second solvent were periodically added to the first solvent, or on a chromatotron. One percent acetic acid was added during the chromatographies of carboxylic acids.

8.1. SYNTHESIS OF 7-CARBOXY-6-FORMYL-4-METHOXY SPIRO[BENZOFURAN-2(DH)-CYCLOHEXANE (62)]

Preparation of 58: A solution of n-butyllithium in hexane (32.67 ml, 65.34 mmol) was added dropwise to a cold solution (0°C) of 3,5-dimethoxybenzyl alcohol (56, 5 g, 29.7 mmol) and TMEDA (5.67 ml, 35.64 mmol) in THF (300 ml). After stirring at 0°C for 15 min and at room temperature for 90 min, the reaction was cooled to -45°C and copper (I) iodide was added (7054 mg, 37.13 mmol). One hour later, the electrophile 57 [prepared as described in J. Heterocyclic. Chem. 26, 879-891 (1989)] was added via syringe. After stirring overnight at room temperature, concentrated ammonium hydroxide (100 ml) was added, and the reaction products were extracted with ethyl acetate (4 x 150 ml). The combined organic phases were dried over MgSO₄, the solvent was evaporated in vacuo and the remaining oil was chromatographed yielding 58 (6678 mg, 25.49 mmol, 86%) as a viscous oil, which crystallized on standing. This was recrystallized (hexane ether) giving a white solid mp. 61-63°C; IR (KBr): 3290, 2930, 1590, 1460, 1425, 1210, 1140, γ 1120 cm⁻¹; ¹H NMR: 1.56 (m, 4H), 1.93 (m, 4H), 2.62 (bs, 1H, exc), 3.26 (bs, 2H), 3.79 (s, 6H), 4.59 (s, 2H), 5.20 (bs, 1H), and 6.54 (s, 1H); ¹³C NMR:

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22.5, 23.1, 25.3, 28.8, 30.6, 55.8, 65.7, 102.5, 116.6, 119.9, 136.3, 139.8, and 158.5; Elemental analysis: calcd. C=73.25, H=8.45; obsd. C=73.21, H=8.52.

Preparation of 59: A solution of n-butyllithium in 5 hexane (4.2 ml, 8.4 mmol) was added to compound 58 (1000 mg, 3.82 mmol) in hexane (50 ml) and TMEDA (0.69 ml, 4.58 mmol). After stirring at 0°C for 15 min and at room temperature for 90 min, the resulting suspension was cooled to -78°C and dry carbon dioxide was bubbled for 1 hr at 10 -78°C and during 1 hr at room temperature. After adding 2N NaOH (25 ml), the unreacted material was extracted with ether and the extracts were discarded. The aqueous phase was acidified with 6N HCl and the reaction products were extraced with ether (4 x 50 ml). After drying ($MgSO_4$) and 15 concentration in vacuo of the organic phase, 59 (727 mg, 2.52 mmol, 66%) was recovered as a solid mp. 103-104.5°C (recrystallized from hexane-ether); IR (KBr): 3000-2820, 1740, 1600, 1460, 1420, 1340, 1240, 1200, 1090, 1015, and 940 cm^{-1} ; 1H NMR: 1.48-2.00 (m, 8H), 3.30 (s, 2H), 3.88 (s, 2H), 4.03 (s, 3H), 5.19 (bs, 1H), 5.19 (s, 2H), and 6.64 (s, 1H); ^{13}C NMR: 22.5, 23.1, 25.3, 28.9, 31.0, 56.2, 62.6, 68.8, 98.7, 109.8, 121.0, 123.0, 136.2, 148.8, 158.4 and 164.3; Elemental analysis: calcd. C=70.83, H=6.94; C=70.77, H=6.97.

25 **Preparation of 60:** A solution of 59 (470 mg, 1.63 mmol) was demethylated until no more starting material was left, according to the TLC. The reaction products were extracted with ethyl acetate (4 x 40 ml), then dried ($MgSO_4$), concentrated in vacuo and purified to yield 60 (332 mg, 1.21 mmol, 74%) as a white solid mp. 150-151.5°C (recrystallized from hexane-ether); IR (KBr): 3420, 3000-2800, 1720, 1600, 1465, 1345, 1280, 1250, 1195 and 1075; 1H NMR: 1.45-2.00 (m, 8H), 3.27 (s, 2H), 3.88 (s, 3H), 5.2 (s, 2H), 6.5 (s, 1H) and 7.71 (s, 1H); ^{13}C NMR:

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22.4, 23.0, 25.2, 28.7, 30.2, 56.2, 70.4, 96.1, 104.2,
115.5, 120.9, 135.4, 146.2, 155.0, 165.2 and 172.8.

Preparation of 61: Amberlyst 15 (290 mg, 2 g/mmol) was added to a solution of 60 (40 mg, 0.146 mmol) in dry 5 methylene chloride (2.9 ml, 20 ml/mmol) after stirring overnight at room temperature, the solvent was filtered through a short column of silica gel, the solids were washed with ethyl acetate and the filtrate was filtered through silica gel. The combined filtrates were concentrated in vacuo and chromatographed, affording 61 (38 mg, 0.139 mmol, 95%) as a solid mp. 132-134°C (recrystallized from hexane-ether); IR (KBr): 2980-2820, 1740, 1610, 1440, 1330, 1280, 1260, 1235, 1205, 1145, 1080, 1010, 930 and 780 cm⁻¹, ¹H NMR: 1.35-2.00 (m, 10H), 2.89 (s, 2H), 3.91 (s, 15 3H), 5.20 (s, 2H) and 6.46 (s, 1H); ¹³C NMR: 23.1, 25.0, 37.1, 55.9, 69.8, 93.7, 96.0, 102.0, 114.8, 150.2, 157.8, 162.1 and 169.6; Elemental analysis: calcd. C=70.04, H=6.62; obsd. C=70.11, H=6.62.

Preparation of 62: Compound 61 (211 mg, 0.77 mmol) was dissolved in THF (4 ml) and the resulting solution was added to a solution of 10N NaOH (6 ml, 60 mmol) and FTBA (1 ml, 1 mmol). Potassium permanganate was (KMnO₄) added to the system until no more starting material was observed by TLC. The excess of KMnO₄ was destroyed with sodium sulfite and the reaction was acidified with 6N H₂SO₄. The organic material was extracted with ethyl acetate (4 x 50 ml), the extracts were dried (MgSO₄), concentrated in vacuo and chromatographed yielding 62 (120 mg, 0.41 mmol, 53%) as a white solid mp. 128-130°C (recrystallized from hexane-ether); IR (KBr): 2950, 2850, 1830, 1770, 1625, 1455, 1340, 1270, 1210, 1145, 990, 985 and 745 cm⁻¹; ¹H NMR: 1.35-1.95 (m, 10H), 2.86 (s, 2H), 3.91 (s, 3H), 5.90 (bs, 1H), 6.53 (s, 1H) and 6.61 (s, 1H); ¹³C NMR: 23.1, 24.9, 37.0, 37.1, 56.0, 93.9, 97.3, 98.0, 102.6, 116.4, 150.1,

157.3, and 162.1 and 167.6; Elemental analysis: calcd. C=66.20, H=6.25; obsd. C=66.03, H=6.18.

5 8.2. SYNTHESIS OF 6-CARBOXY-7-FORMYL-4-METHOXY-SPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE (68) AND
10 6,7-DICARBOXYL-4-METHOXYSPIRO[BENZOFURAN-2(3H)-CYCLOHEXANE] (66)

15 Preparation of 65: A solution of n-butyllithium in hexane (2.20 ml, 4.95 mmol) was added to a solution of N,N,N',N'-trimethylethylenediamine (0.65 ml, 5.09 mmol) in THF (6 ml) at -20°C (CCl₄/CO₂). After 30 min, 11b (1170 mg, 4.76 mmol, prepared as described in section 6.8 *supra*) in THF (4 ml) was added dropwise, followed 30 min later by n-BuLi (6.35 ml, 14.28 mmol). The resulting system was kept at -20°C for 24 h, and DMF was added (02.20 ml, 28.56 15 mmol). After 24 h reaction period, the reaction products were partitioned between ether (4 x 50 ml) and brine (50 ml), chromatography of the extracts gave 65 (1135 mg, 4.14 mmol, 87%) as a solid mp. 129-131°C (recrystallized from hexane-ether); IR (KBr): 3000-2840, 1670, 1600, 1470, 20 1425, 1390, 1320, 1280, 1260, 1210, 1130, 1030, 890, 850, 770, 700 and 620 cm⁻¹; ¹H NMR: 1.40-1.93 (s, 10H), 2.93 (s, 2H), 3.94 (s, 3H) 7.04 (s, 1H), 10.35 (s, 1H) and 10.70 (s, 1H); ¹³C NMR: 22.9, 24.9, 37.2, 37.7, 56.0, 93.0, 103.6, 113.7, 120.2, 138.7, 160.3, 164.7, 188.6 and 192.6;
25 Elemental analysis: calcd. C=70.06, H=6.61; obsd. C=69.84, H=6.65.

30 Preparation of 68: A 4 N solution of potassium hydroxide (52.92 mmol) was added dropwise to a stirred solution of compound 65 (2900 mg, 1058 mmol) in THF (10 ml) and water (7 ml) containing dissolved silver nitrate (3777 mg, 22.22 mmol, 1.05 eq.) at room temperature. The reaction system was protected from direct light. After stirring for 2 h at room temperature, the solids were filtered and extensively washed with distilled water. 2N
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H₂SO₄ was added until pH 3, and the reaction products were extracted with ether (3 x 150 ml), washed with brine (2 x 25 ml) and dried (MgSO₄). Chromatography of the reaction products allowed the recovery of the starting material 5 (1823 mg, 63%), compound 68 (326 mg, 1.12 mmol, 11%, 29% corrected yield) and compound 66 (614 mg, 2.01 mmol, 19%, 51%). Compound 68: mp: 148-150°C (rec. from hexane-ether); IR (KBr): 3440, 3000-2840, 1740, 1630, 1450, 1345, 1290, 1270, 1150, 1105, 1010, 910, 860, 770, 690, cm⁻¹; ¹H NMR (DMSO d₆): 1.30-1.52 (m, 4H), 1.62-1.81 (m, 6H), 2.91 (s, 2H), 3.87 (s, 3H), 6.55 (bs, 1H), 6.85 (s, 1H), and 7.95 (bs, 1H); ¹³C NMR (DMSO d₆): 22.52, 24.47, 36.57, 37.56, 55.86, 91.90, 96.33, 98.34, 120.60, 121.48, 128.99, 154.0-3, 158.28, 168.42; MS (e/m, %): 290 (M+, 89), 289 (72), 15 272 (100), 271 (88), 244 (65), 215 (78), 192 (98), 191 (95), 190 (65), 165 (93), 164 (82), 79 (89). Elemental analysis: calcd. C=66.20, H=6.25; obsd. C=66.19, H=6.26.

Direct Preparation of 66: To a solution 65 (153 mg, 0.56 mmol) in ethanol (5 ml), was added a solution of 20 silver nitrate (222 mg, 1.30 mmol) in distilled water (1 ml), followed by KOH (3 ml, 2.99 mmol). The system was stirred overnight at room temperature, shielded from the light, then it was filtered and the residue was carefully washed with water. The combined aqueous phases were 25 extracted with ether, the aqueous phase was then acidified and extracted with ether (3 x 25 ml); the combined organic phases were dried and chromatographed to afford 66 (156 mg, 0.51 mmol, 91%) as a white solid mp. 188-190°C (recrystallized from acetone); IR (KBr): 3500-2400, 3000-2850, 30 1700, 1610, 1410, 1330, 1290, 1130, 1040, 1000, 930, 855, 750 and 660 cm⁻¹; ¹H NMR: (acetone d₆): 1.40-1.90 (m, 10H), 2.93 (s, 2H), 3.91 (s, 3H) and 6.95 (s, 2H); ¹³C NMR: (acetone d₆): 23.5, 25.6, 37.6, 38.7, 56.1, 91.7, 105.1, 111.6, 118.8, 133.0, 157.8, 158.8, 167.2 and 186.1; Ele- 35

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mental analysis: calcd. C=62.74, H=5.92; obsd. C=62.66, H=6.01.

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8.3. SYNTHESIS OF 4-METHOXY-5a,6,8,8a-TETRAHYDROSPIRO[BENZO[2,1-b:3,4-c']DIFURAN-2(8H) CYCLOHEXAN]-6,8-DIONE; 6,7-DIHYDROXYMETHYL-4-METHOXYSPIRO[BENZOFURAN-2(3H) CYCLOHEXANE]; AND 4-METHOXYSPIRO[BENZO[2,1-B:3,4-C']DIFURAN-2(3H)-CYCLOHEXAN]-6(8H)-ONE

Preparation of 63: A cold and stirred solution of 62 (150 mg, 0.52 mmol) in acetone (10 ml) was treated with excess of Jones reagent (Bowden et al., 1946, J. Chem. Soc. 39; Bowers et al., 1953, J. Chem. Soc., 2548) until the starting material was completely converted (TLC). The excess reagent was destroyed with isopropanol, brine was added (30 ml) and the reaction products were extracted with ethyl acetate (4 x 50 ml) the extracts were dried ($MgSO_4$), concentrated in vacuo and chromatographed yielding 63 (134 mg, 0.46 mmol, 89%) as a white solid mp. 197-198.5°C (recrystallized from acetone); IR (KBr): 3400, 2940, 2860, 1740, 1620, 1450, 1330, 1235, 1140, 1090, 1020, 930 and 760 cm^{-1} ; 1H NMR (acetone d_6): 1.45-1.95 (m, 10H), 3.03 (s, 2H), 4.08 (s, 3H), 7.15 (s, 1H); ^{13}C NMR (acetone d_6): 23.5, 25.5, 37.7, 38.3, 57.2, 95.6, 102.1, 123.9, 134.9, 158.4, 161.2, 163.8 and 164.2; MS (m/z, %): 288 (M+, 40), 287 (32), 231 (40), 208 (49), 207 (52), 85 (27), 81 (100), 77 (36), 71 (28), 69 (42), 57 (67) and 55 (61); HRMS: expected 288.0998 for $C_{16}H_{16}O_5$, obsd.: 288.0999.

Preparation of 64: A solution of 61 (300 mg, 1.09 mmol) in THF (15 ml) was treated for 3 h with LAH (slight excess). After adding $Na_2SO_4 \cdot 10H_2O$ and ether, the reaction products were filtered, affording the diol 64 (260 mg, 0.94 mmol, 86%); 1H NMR: 1.40-1.90 (m, 10H), 2.93 (s, 2H), 3.83 (s, 3H), 4.66 (s, 2H), 4.70 (s, 2H) and 6.47 (s, 1H).

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Preparation of 67: To a solution of 64 (260 mg, 0.935 mmol) in dry methylene chloride (15 ml) BaMnO₄ (1197 mg, 4.68 mmol) was added and the resulting suspension was stirred for 3 days at room temperature. After recovering 5 the reaction products by partition between brine (30 ml) and ethyl acetate (3 x 50 ml) it was observed that this was an equimolecular mixture of 67 and 65.

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9. COMPLEMENT INHIBITION BY DI- AND
TRI- SUBSTITUTED SPIRO[BENZOFURAN-
2(3H)-CYCLOHEXANES]

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The 4 substituted spirobenzofuran compounds of example 7 and the disubstituted spirobenzofuran compounds of example 8 were tested for their capacity to inhibit complement-mediated lysis of sheep red blood cells (SRBC) as described in Section 6.6.2 supra with the modification that the human serum used as the complement source was diluted to 1 to 100 in Hepes buffer, instead of 1 to 125. The results of the hemolysis assays are shown in FIGURE 8 and Tables XI and XII.

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Table XI

Inhibition of Hemolysis by 6,7-disubstituted
-4-methoxy spiro[benzofuran-2(3H)-cyclohexanes]

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	Compound	Mean mM	IH ₅₀ (± SD)*	n=
10	11a	1.330	(± 0.490)	10
	44a	0.530	(± 0.190)	23
	62	1.670	(± 0.153)	3
	66	0.800	(± 0.356)	3
15	68	0.164	(± 0.076)	7
	K76COOH	0.570	(± 0.170)	9

* The concentration of compound (± standard deviation)
 required to produce a value for hemolysis inhibition of
 20 0.5 as described in Section 6.36.2 supra.

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Table XII

Inhibition of Hemolysis by
 6-carboxyl-4-substituted
spiro[benzofuran-2(3H)-cyclohexanes]

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	Compound	Mean mM	IH ₅₀ (± SD)*	n=
10	31a	1.45	(± 0.21)	2
10	44a	0.532	(± 0.193)	29
10	44b	0.580	(± 0.216)	3
15	55a	2.53	(± 1.00)	2
15	55b	0.430		1
15	55c	0.280	(± 0.014)	2
15	55d	>2.8		1
15	55e	0.305	(± 0.049)	2
15	55g	2.320	(± 0.099)	2
20	55h**	0.320	(± 0.056)	2
20	55i	1.45	(± 0.44)	2
20	K76COOH	0.570	(± 0.170)	9

* The concentration of compound (± standard deviation) required to produce a value for hemolysis inhibition of 0.5 as described in Section 6.36.2 supra.

** The sodium salt of 4-p-aminophenoxy-carboxy spiro[benzofuran-2(3H)-cyclohexane]

30 By comparing the inhibition of hemolysis by compounds 62, 66 and 68 as shown in figure 8 and Table XI, it can be seen that the particular arrangement of substitutents at positions 6 and 7 of the benzofuran ring is important for anti-hemolytic activity. By comparing IH₅₀ values for the 35 position 4-substituted series as shown in Table XII' (for

example, the values for 55c, 55e, and 55h), improvements in anti-hemolytic activity can also be obtained through the optimal choice of the R substituent at position 4. In addition, several of the compounds (see for example, 5 compounds 55c, 55e, 55h and 68 in particular) are more effective in inhibiting complement-mediated hemolysis than is K76COOH. It is anticipated that optimal substituents at position 4, combined with an optimal pair of substituents at position 6 and 7, will result in even more 10 potent complement inhibitors.

10. IN VITRO AND IN VIVO INHIBITION
OF COMPLEMENT BY DI- AND TRI-SUBSTITUTED
SPIRO[BENZOFURAN-2(3H)-CYCLOHEXANES]

15 10.1. INTRODUCTION

The role of complement in hyperacute graft rejection has been demonstrated in animal models of both sensitized allograft (Knechtle et al., J. Heart Transplant., 1983, 4:541; Pruitt and Bollinger, J. Surg. Res., 20 1991, 50:350-355; as well as xenograft transplantation (Adachi et al., Transplant Proc., 1987, 119:1145-1148; Migagawa et al., Transplantation, 1988, 46:825; Prewitt et al., 1991, Transplantation 52:868-873). We have chosen an established discordant xenograft model, a guinea pig-to- 25 rat cardiac transplant, to assess the effects of the substituted dihydrobenzofurans on hyperacute rejection in vivo.

30 10.2. INHIBITION IN VITRO OF RAT AND HUMAN COMPLEMENT

Compounds 68 and 44a were prepared and characterized as described in sections 8.2 and 7.1, respectively. These compounds were demonstrated to inhibit complement-mediated hemolysis using human serum as a complement source, as described in section 6.6.2 and Section 9, 35 supra. Compounds 68 and 44a were also demonstrated to

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inhibit rat complement using the methods of section 6.6.2, except that rat serum was substituted for human serum as the complement source. A soluble human recombinant complement receptor type 1 (sCR1) was prepared as described 5 (Weisman et al., Science, 1990, 249:146-151; Yeh et al., J. Immunol., 1991; and International Patent Publications No. WO 89/09220 and WO 9/05047, both entitled "The human C3b/C4b receptor (CR1)" Fearon et al.) and used as a positive control the in vitro assays. The results of 10 these studies are summarized in Table XIII.

Table XIII

Inhibition of rat or human complement-mediated hemolysis by substituted spiro[benzofuran-2(3H)- 15 cyclohexanes] and by sCR1

Compound	IH ₅₀ * for Human Complement	IH ₅₀ for Rat Complement
sCR1	0.9 nM	1.5 nM
68	100 μM 164 μM	2500 μM 11,000 μM
44a	538 μM	17,300 μM

*The concentration of compound required to produce a value 25 for hemolysis inhibition of 0.5 as described in Section 6.6.2, supra.

As seen in Table XIII, the human sCR1 protein inhibits 30 its rat and human complement with similar potency in this assay. The compounds, on the other hand, more effectively inhibit human than rat complement. Twenty-five- to 70-fold higher concentrations of compound 68 were required to inhibit rat complement-mediated lysis than were required to inhibit human complement-mediated lysis to similar 35 levels.

10.3. INHIBITION OF ALTERNATIVE PATHWAY-MEDIATED HEMOLYSIS

The inhibition of sheep red blood cell lysis as described in section 6.6.2 predominantly reflects on 5 classical complement pathway activation since it is initiated by the antigen-antibody complexes on the surface of the sensitized erythrocytes. The assay probably includes a minor contribution from alternative pathway activation as well.

10 The capacity of the substituted dihydrobenzofur-
ans to inhibit the alternative complement pathway was
assessed using the modified method of Platts-Mills and
Ishizaka, 1974, J. Immunol., 113:348-358. Essentially,
rabbit erythrocytes were lysed using human serum as com-
15 plement in gelatin veronal buffer (GVB, Sigma, St. Louis,
MO) with added EGTA (ethylene glycol-bis[β -aminoethyl
ether]N,N,N¹,N¹-tetraacetic acid) and Mg²⁺ to 8 mM and 2 mM,
respectively. Rabbit erythrocytes (4.5×10^6 cells/ml),
human serum (1 in 24 dilution), and the compounds to be
20 tested were incubated one hour at 37°C in a V-bottom
microtiter plate, cells pelleted by centrifugation, the
supernatants transferred to a flat-bottom microtiter
plate, and the absorbance at 410 nm determined. Samples
were paired with identical controls lacking human serum
25 (complement-independent lysis). Both samples and controls
were run in triplicate. Control values were subtracted
from sample values and the fractional inhibition was
determined relative to the uninhibited (no added compound)
sample. Results are reported as the concentration of
30 compound yielding fifty per cent (50%) inhibition, AH₅₀.
The AH₅₀ values for several of the compounds are included
along with the results of other in vitro assays in Table
XIV. As seen in Table XIV, compound 68 inhibited the
classical pathway more effectively than K76COOH, but was
35 somewhat less potent than K76COOH in inhibiting alterna-

-92-

tive complement pathway. Compound **44a** inhibited the classical pathways as well as K76COOH, but was clearly less potent than K76COOH or **68** in inhibiting the alternative pathway.

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10.4. INHIBITION OF C3b PROTEOLYSIS BY FACTOR I

K76COOH has been shown to inhibit the proteolysis of C3b by factor I and the cofactor protein H (Hong et al., 1981, J. Immunol., 127:104-108. The capacity of the substituted dihydrobenzofurans to inhibit the specific degradation of the C3b α -chain by factor I was assessed on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Wong et al., 1985, J. Immunol. Methods, 82:303-313; Weisman et al., 1990, Science, 249:146-151) using either factor H or sCR1 as cofactor. Purified C3b at 1.2 μ M, factor I (Cytotech, San Diego, CA) at 0.1 μ M, sCR1 at 8 nM as a cofactor, and test compound at varying concentrations, were incubated for 60 minutes at 37°C. The reactions were stopped by the addition of reducing SDS-PAGE sample buffer (Laemmli, 1970, Nature, 227:680-685) subjected to electrophoresis on 4-20% polyacrylamide gradient gels, visualized with Coomassie Blue stain, and quantitated by densitometry. Controls lacking factor I and controls with factor I but lacking test compounds were included. Results are reported as the concentration of test compound which inhibited approximately fifty percent (50%) of the C3b α -chain degradation, IC₅₀.

The results for several compounds are included in Table XIV.

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TABLE XIV

In vitro assays of complement inhibition by substituted spiro[benzofuran-2(3H)-cyclohexanes].
 5 Concentration* (μM) yielding 50% inhibition.

Compound	Classical pathway IH_{50} (μM)	Alternative pathway AH_{50} (μM)	C3b cleavage by Factor I (μM)
K76COOH	570 (± 170 ; n=9)	850 (n=1)	110
68	160 (± 69 ; n=9)	1360 (± 320 ; n=3)	150
44a	530 (± 190 ; n=23)	>5030	2740

15 *Values reported are the mean (\pm standard deviation; sample number, n)

20 Compound 68 and K76COOH were equally effective in inhibiting proteolysis of C3b by factor I using sCR1 as a cofactor.

10.5. GUINEA PIG-TO-RAT CARDIAC TRANSPLANT

The effects of compounds 68 and 44a on hyperacute graft rejection were tested in a model of discordant 25 xenografting. The methods were the same as described previously (Prewitt et al., 1991, Transplantation 52:868-873) except that the test compounds were substituted for the active agent (sCR1) of the earlier study. As indicated in the earlier study, male Lewis rats, 12-16 weeks of age, and male Hartley guinea pigs, 4-10 weeks of age 30 (Charles River, Wilmington, MA) served as cardiac xenograft recipients and donors, respectively. Under Halothane anesthesia, heterotopic cardiac xenotransplantation 35 was performed by a modification of the microvascular

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technique of Ono and Lindsey, 1990, J. Thorac. Cardiovas. Surg., 57:225, with anastomosis of the donor aorta to the recipient infrarenal aorta and the donor pulmonary artery to the recipient inferior vena cava. Cardiac xenografts 5 were evaluated visually over the first 30 minutes following reperfusion and then, following abdominal closure, every 10 minutes by palpation until rejection. Rejection was defined as total cessation of cardiac xenograft contraction and was confirmed by direct visualization and 10 histological examination. The compounds 68 and 44a were prepared as 14 mg/ml solutions in phosphate buffered saline (PBS) and were administered as a 2.0 ml intravenous bolus into the recipient inferior vena cava, superior to the caval cross clamp, immediately prior to cardiac xenograft reperfusion. Control animals received similar 15 volumes of PBS. Three (n=3) control animals were tested concurrent with the animals receiving compound 68; data from historic controls (n=13) subjected to the same procedure and previously reported (Pruitt et al., 1991, Transplantation 52:868-873) are also included for comparison. 20 Table XV includes the xenograft survival times (minutes) for individual animals and the means and standard errors of the mean (SEM) for the various groups. As can be seen in the table, compound 68 treatment resulted in prolonged 25 graft survival times in 2 of the 3 animals tested relative to controls.

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TABLE XV
Guinea pig-to-rat cardiac transplant

Treatment	Graft survival time (minutes)	Mean \pm SEM (minutes)
Historic controls (PBS)	5, 7, 7, 9, 9, 10, 10, 11, 16, 17, 24, 40, 60	17 \pm 4
Concurrent controls (PBS)	5, 7, 19	10 \pm 4
68 at 28 mg/rat	7, 150, 270	142 \pm 76
44a at 28 mg/rat	7, 9	8

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The present invention is not to be limited in scope by the foregoing examples, which are intended as purely illustrative of specific aspects of the invention and any compounds that are functionally equivalent are within the 5 scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope 10 of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entireties.

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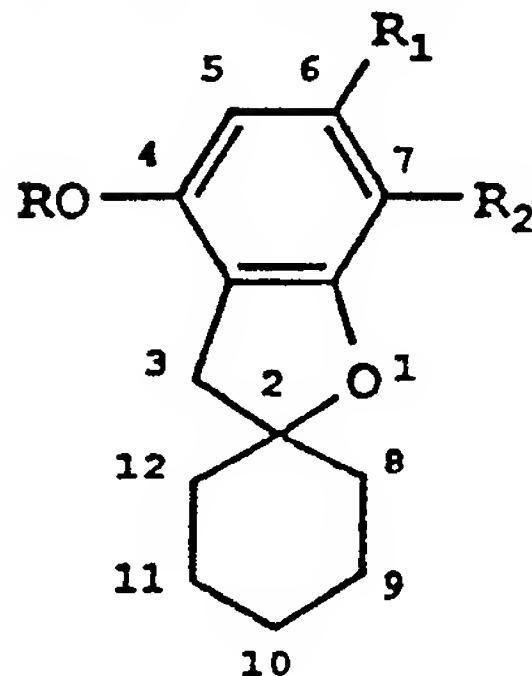
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WHAT IS CLAIMED IS:

1. A compound of the general formula, 4:

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in which R represents a hydrogen atom, a lower alkyl group, a substituted lower alkyl group, a benzyl group, a substituted benzyl group, a phenyl group or a substituted phenyl group; R₁ and R₂ represent independently, a carboxylic acid group, a formyl group, a hydroxymethyl group, a N-(lower alkyl)carbamoyl group, a trifluoroacetyl group, a halide group, a vinyl group, a substituted vinyl group having up to 10 carbon atoms, an alkylidene group having up to 20 carbon atoms, an aliphatic acyl group, a substituted aliphatic acyl group, an aromatic acyl group, a substituted aromatic acyl group, a sulfamoyl group, an aminomethyl group, a N-(lower alkyl)aminomethyl group, a N,N-di(lower alkyl)aminomethyl group, a heterocyclic ring, an N-(acyl)carbamoyl group, an amidino group or a hydrazide group; R₁ and R₂ together with the carbon atoms to which they are attached may also represent a cyclic anhydride or lactone; or a pharmaceutically acceptable acid or base addition salt or ester thereof.

2. The compound of claim 1 which is selected from the group consisting of 6,7-dicarboxy-4-methoxyspiro[benzofuran-2(3H)-cyclohexane], 6,7-diformyl-4-methoxyspiro[benzofuran-2(3H)-cyclohexane], 6,7-

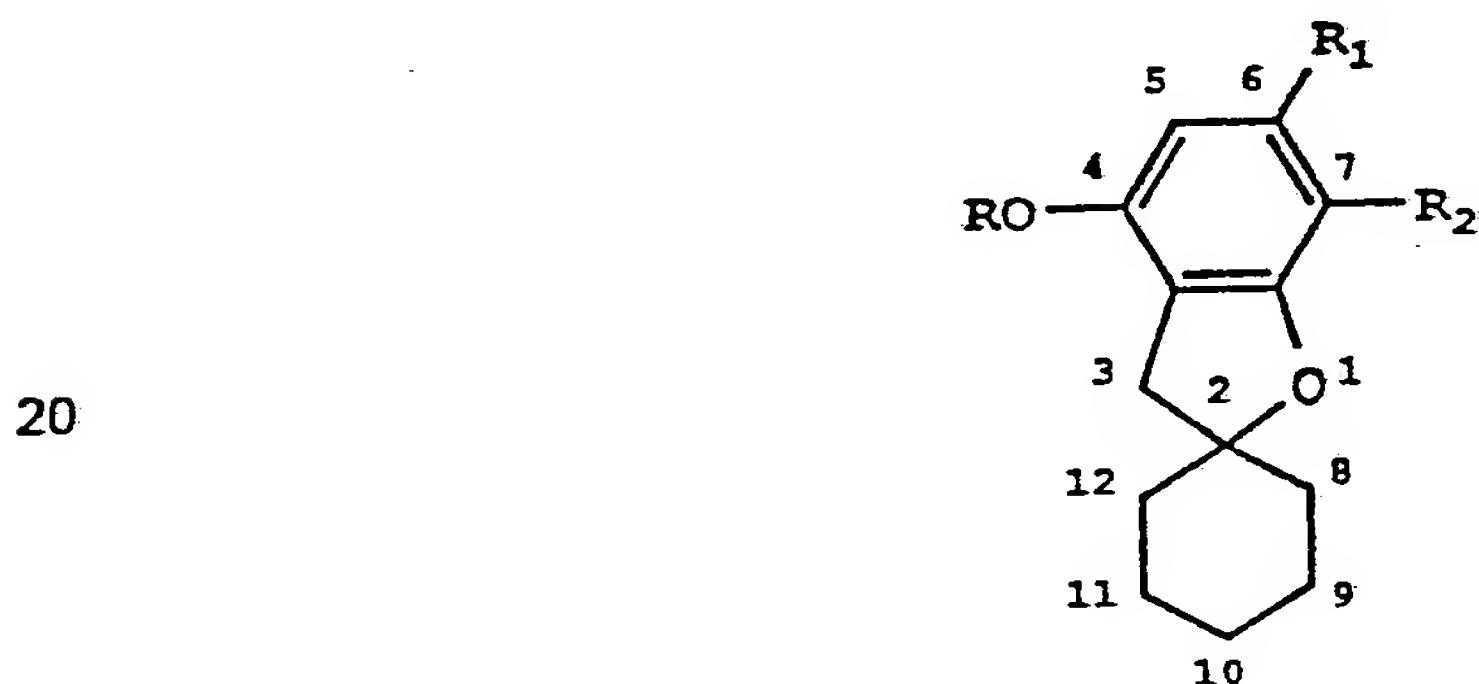
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di(hydroxymethyl)-4-methoxyspiro(benzofuran-2(3H)-cyclohexane], 4-methoxy-3,6-dihydrospiro[benzo[2,1-b:3,4-c']difuran-2(8H)-cyclohexane]-8-one, 7-carboxy-6-formyl-4-methoxyspiro [benzofuran-2(3H)-cyclohexane], 4-methoxy,5a,6,8,8a-tetrahydrospiro[benzo[2,1-b:3,4-c']difuran-2(8H)-cyclohexan]-6,8-dione, and 6-carboxy-7-hydroxymethyl-4-methoxyspiro[benzofuran-2(3H)-cyclohexane] lactone.

10 3. The compound of claim 1 which is selected from the group consisting of 6-carboxy-7-formyl-4-methoxyspiro[benzofuran-2(3H)-cyclohexane] and 6-carboxy-7-formyl-4-phenoxySpiro[benzofuran-2(3H)-cyclohexane].

15 4. The compound of the general formula, 4:



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in which R represents a hydrogen atom, a lower alkyl group, a substituted lower alkyl group, a benzyl group, a substituted benzyl group, a phenyl group or a substituted phenyl group; R₁ represents a carboxylic acid group, or a bioisosteric acid or base group; R₂ represents a formyl group or a bioisosteric neutral group; or a pharmaceutically acceptable acid or base addition salt or ester thereof.

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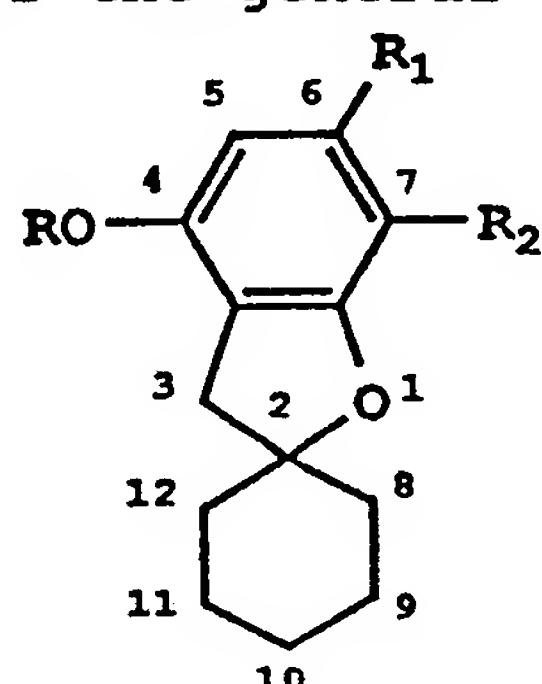
-99-

5. The compound of claim 4 in which said bioisosteric base group is a carbamoyl group, a sulfamoyl group, an N-acylcarbamoyl group or a tetrazole ring.

10 6. The compound of claim 4 in which said bioisosteric base is an aminomethyl group, a N-(lower alkyl)aminomethyl group, a N,N-di(lower alkyl)aminomethyl group, an oxazoline ring, an amidino group or a hydrazide group.

15 7. The compound of claim 4 in which said bioisosteric neutral group is an aliphatic acyl group, a substituted aliphatic acyl group, an aromatic acyl group, or a substituted aromatic acyl group.

20 8. A compound of the general formula, 4:



25 4
in which R represents a hydrogen atom, a lower alkyl group, a substituted lower alkyl group, a benzyl group, a substituted benzyl group, a phenyl group or a substituted phenyl group; R₂ represents a hydrogen atom; R₁ represents
30 a formyl group, carboxylic acid group; or a pharmaceutically acceptable acid or base addition salt or ester thereof.

35 9. The compound of claim 8 which is 6-carboxy-4-methoxyspiro[benzofuran-2(3H)-cyclohexane].

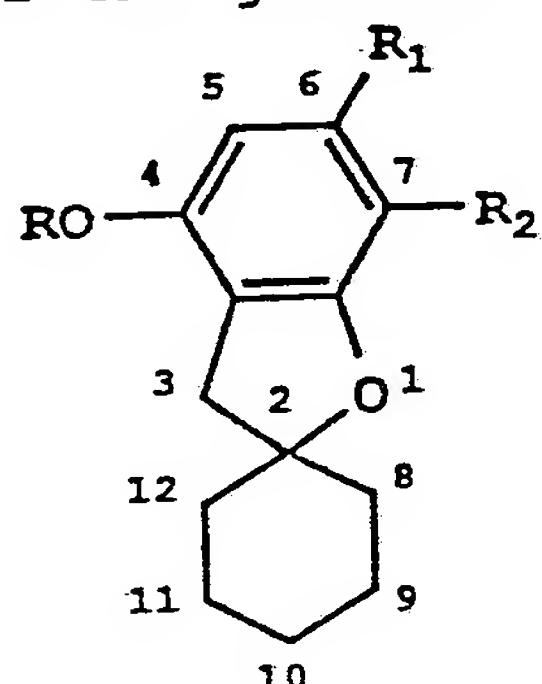
-100-

10. The compound of claim 8 which is selected from the group consisting of 6-carboxy-4-ethoxyspiro[benzofuran-3(3H)-cyclohexane], 6-formyl-4-hydroxyspiro[benzofuran-2(3H)-cyclohexane], 4-benzyloxy-6-
 5 formylspiro[(benzofuran-2(3H)-cylcohexane], 4-butyloxy-6-formylspiro[benzofuran-2(3H)-cyclohexane], 6-formyl-4-formylspiro[benzofuran-2(3H)-cyclohexane], 6-formyl-4-(2-hydroxyethyloxy)spiro[benzofuran-2(3H)-cyclohexane], 6-formyl-4-(p-nitrophenoxy)spiro[benzofuran-2(3H)-
 10 cyclohexane], 6-formyl-4-(p-formylphenoxy)spiro[benzofuran-2(3H)-cyclohexane], 6-formylspiro[benzofuran-2(3H)-cylohexane], 4-benzyloxy-6-carboxyspiro[benzofuran-2(3H)-cyclohexane], 4-butyloxy-6-carboxyspiro[benzofuran-2(3H)-cyclohexane], 6-carboxy-4-phenoxySpiro[benzofuran-
 15 2(3H)-cyclohexane], 6-carboxy-4-(2-hydroxyethyloxy)spiro[benzofuran-2(3H)-cyclohexane], 6-carboxy-4-(p-nitrophenoxy)spiro[benzofuran-2(3H)-cyclohexane], 6-
 carboxyspiro[benzofuran-2(3H)cyclohexine], 6-carboxy-4-(p-aminophenoxy)spiro[benzofuran-2(3H)-cyclohexane, and 6-
 20 carboxy-4-(p-carboxyphenoxy)spiro[benzofuran-2(3H)-cyclohexane].

11. A compound of the general formula, 4:

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in which R represents a hydrogen atom, a lower alkyl group, a substituted lower alkyl group, a benzyl group, a
 35 substituted benzyl group, a phenyl group or a substituted

phenyl group; R₁ and R₂ represent independently a hydrogen atom, a carboxylic acid group, a formyl group, a hydroxymethyl group, a N-(lower alkyl)carbamoyl group, a trifluoroacetyl group, a halide group, a vinyl group, a 5 substituted vinyl group having up to 10 carbon atoms, an alkylidene group having up to 20 carbon atoms, an aliphatic acyl group, a substituted aliphatic acyl group, an aromatic acyl group, a substituted aromatic acyl group, a sulfamoyl group, an aminomethyl group, a N-(lower 10 alkyl)aminomethyl group, a N,N-di(lower alkyl)aminomethyl group, a heterocyclic ring, an N-(acyl)carbamoyl group, an amidino group or a hydrazide group; R₁ and R₂ together with the carbon atoms to which they are attached may also 15 represent a cyclic anhydride or lactone; or a pharmaceutically acceptable acid or base addition salt or ester thereof, provided that R₁ and R₂ do not both represent 20 hydrogen atoms.

12. A pharmaceutical composition for use in a method 20 of treating a patient with an immune disorder or a disorder involving undesirable or inappropriate complement activity comprising an effective amount of the compound of claim 1, 3, 4, 8, 9 or 11.

25 13. The composition of claim 12 in which the immune disorder or disorder involving undesirable or inappropriate complement activity is selected from the group consisting of soft tissue destruction due to burn, myocardial infarct induced trauma, adult respiratory 30 distress syndrome, and myocardial ischemia and reperfusion; specific and non-specific proteolytic processing of C5; inflammation associated with kidney stones, systemic lupus erythematosis, nephrotoxic glomeronephritis, and multiple sclerosis; atrophic gastritis, thyroiditis, allergic 35 encephalomyelitis, gastric mucosa, thyrotoxicosis,

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autoimmune hemolytic anemia, pemphigus vulgaris,
sympathetic ophthalmia, delayed-type hypersensitivity,
autoimmune disorders and drug allergies; and tissue
plasminogen activator therapy and cardiopulmonary bypass.

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14. A pharmaceutical composition for use in a method
of treating a patient with graft rejection, graft-host
reaction, or organ transplant rejection comprising an
effective amount of the compound of claim 1, 3, 4, 8, 9 or
10 11.

15. The composition of claim 15 in which the graft
is a xenograft or an allograft.

15 16. The composition of claim 15 in which the graft
is a heart transplant.

20

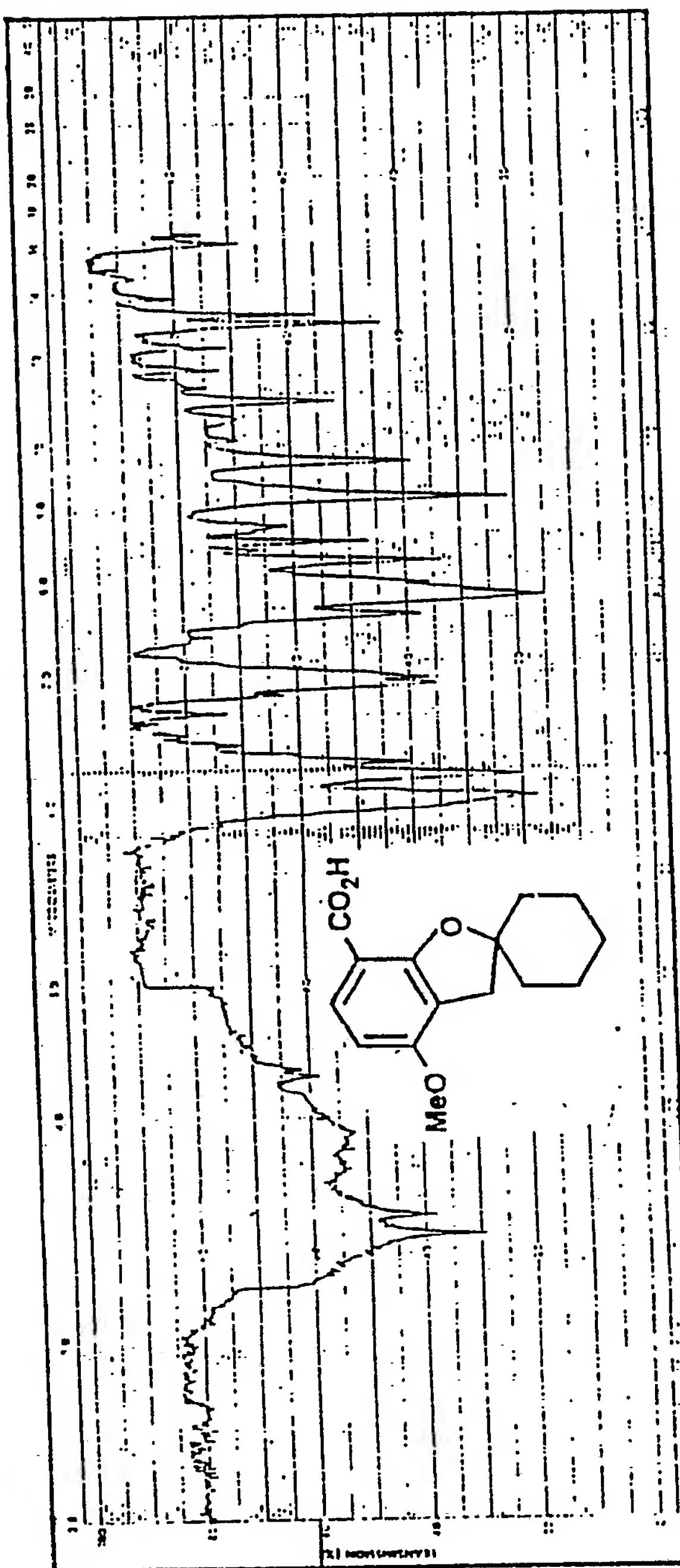
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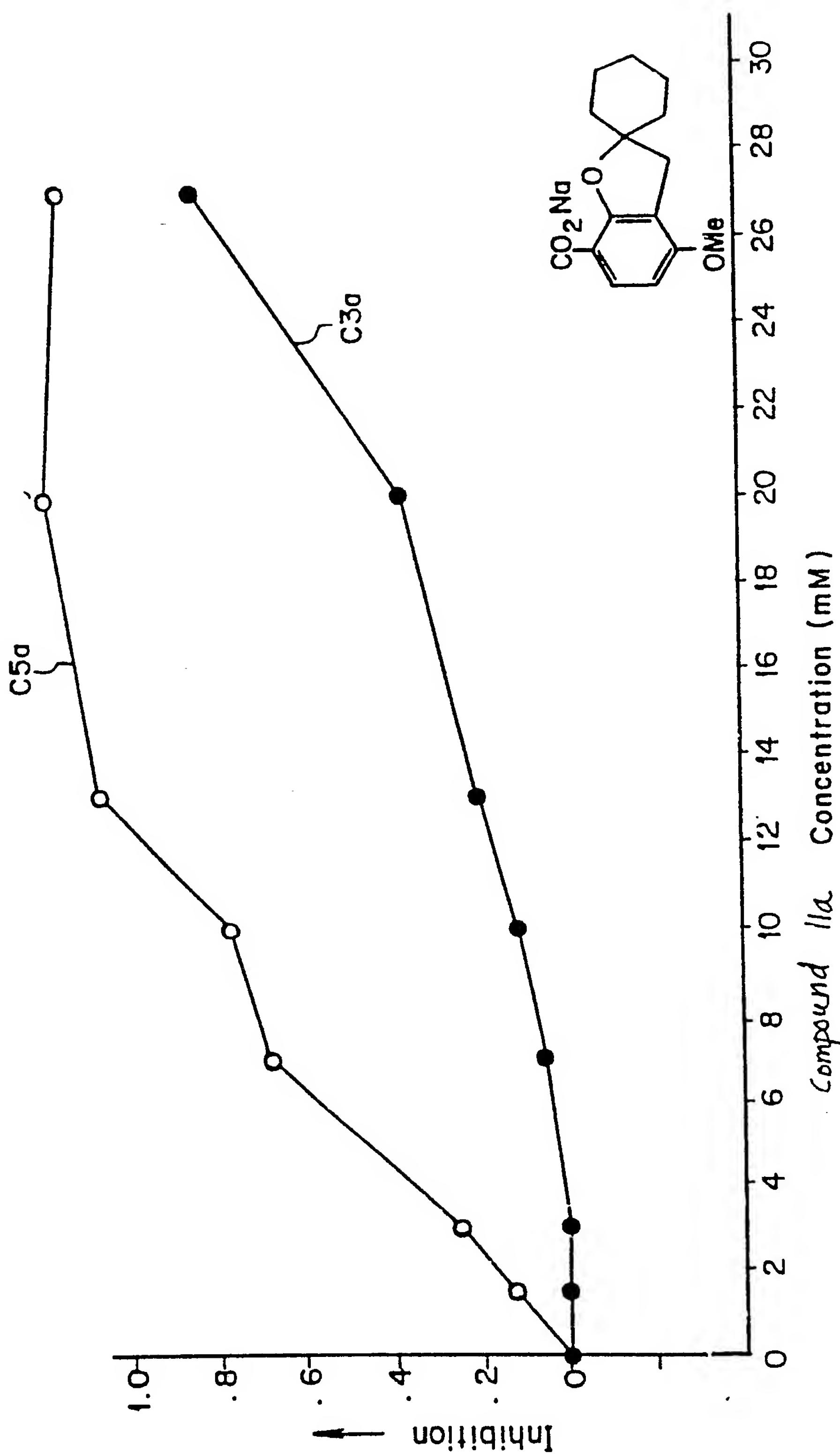
118

Figure 1



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Figure 2



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FIGURE 3
Inhibition of Complement Mediated Hemolysis

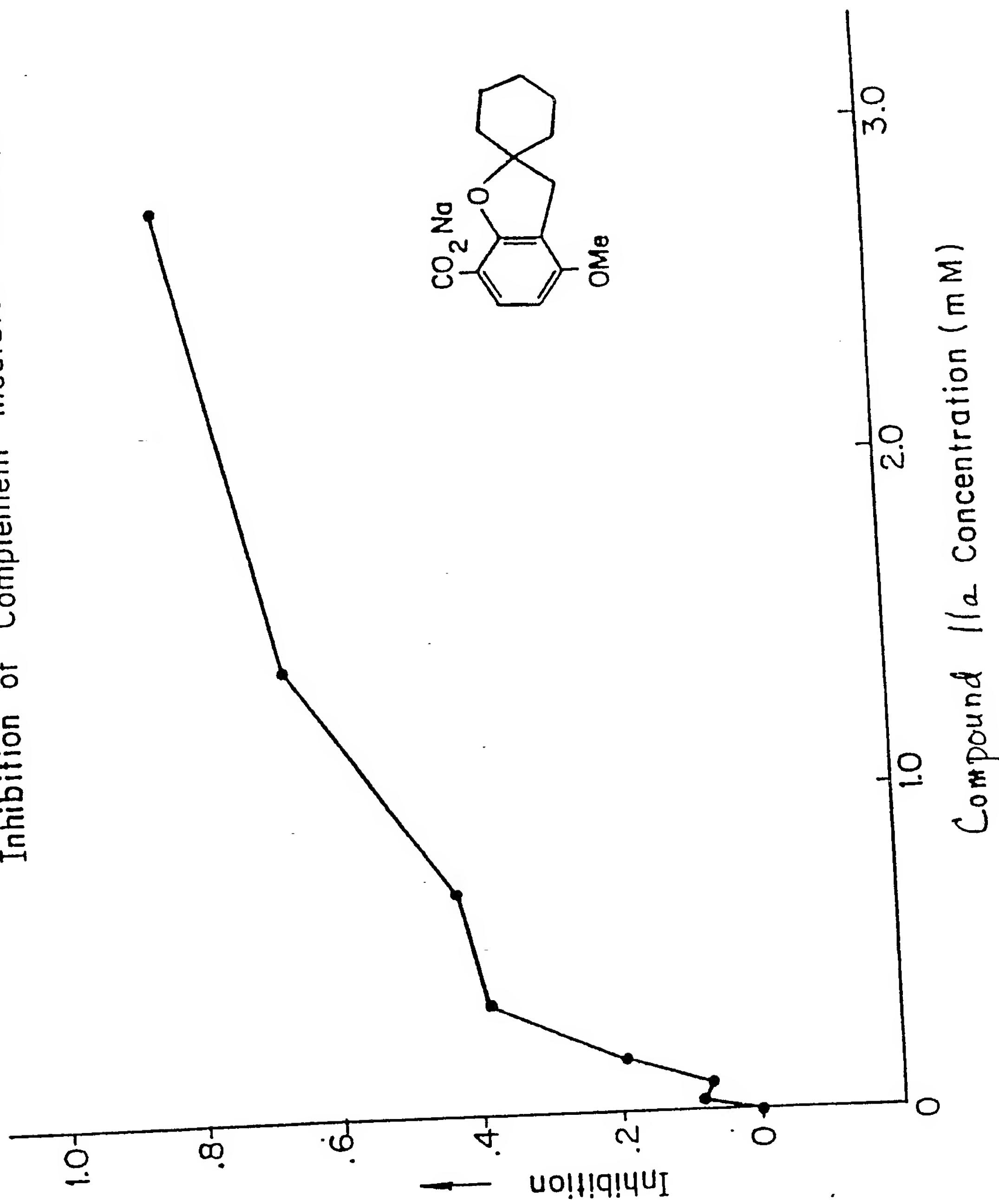
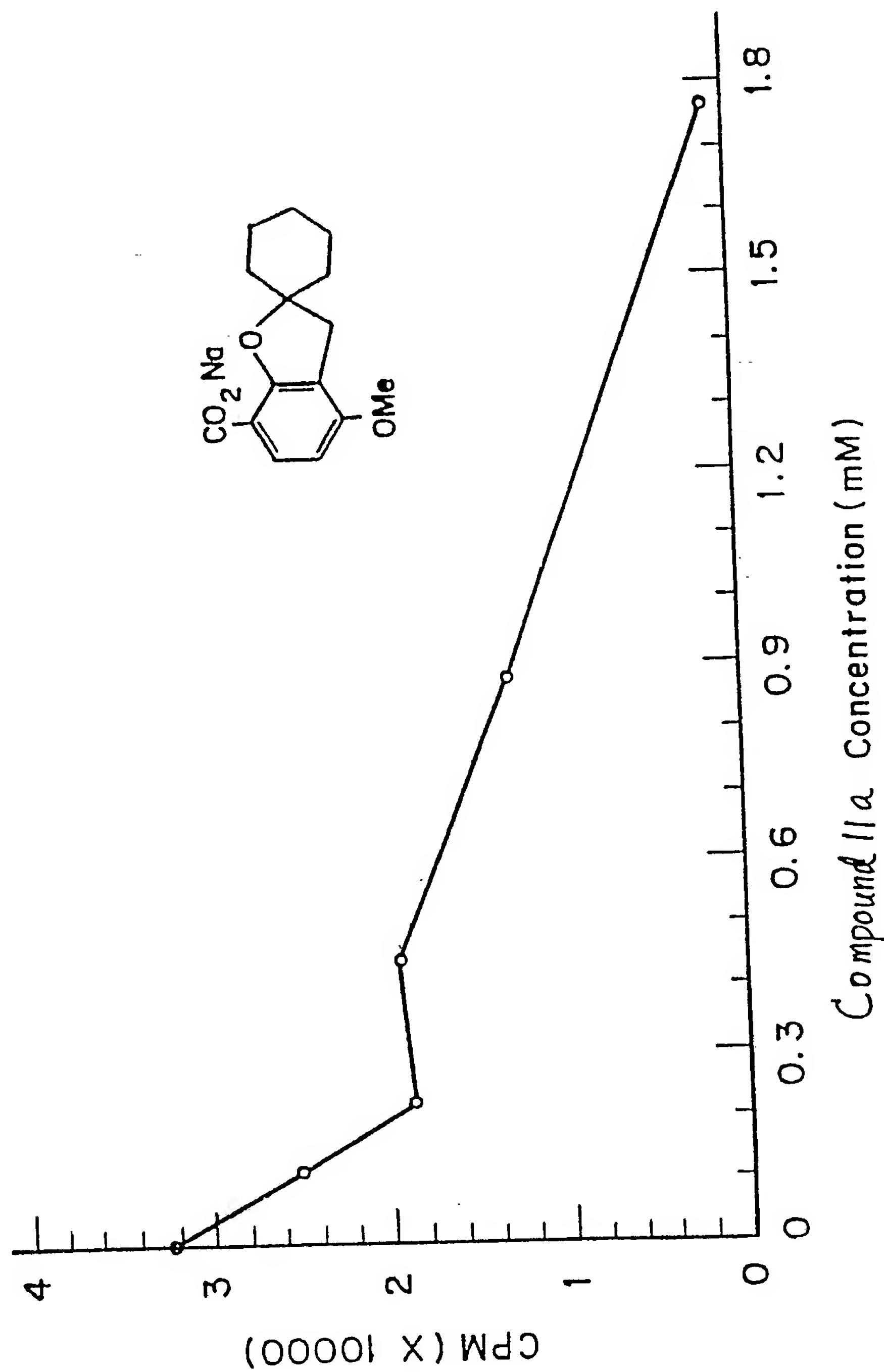


Figure 4

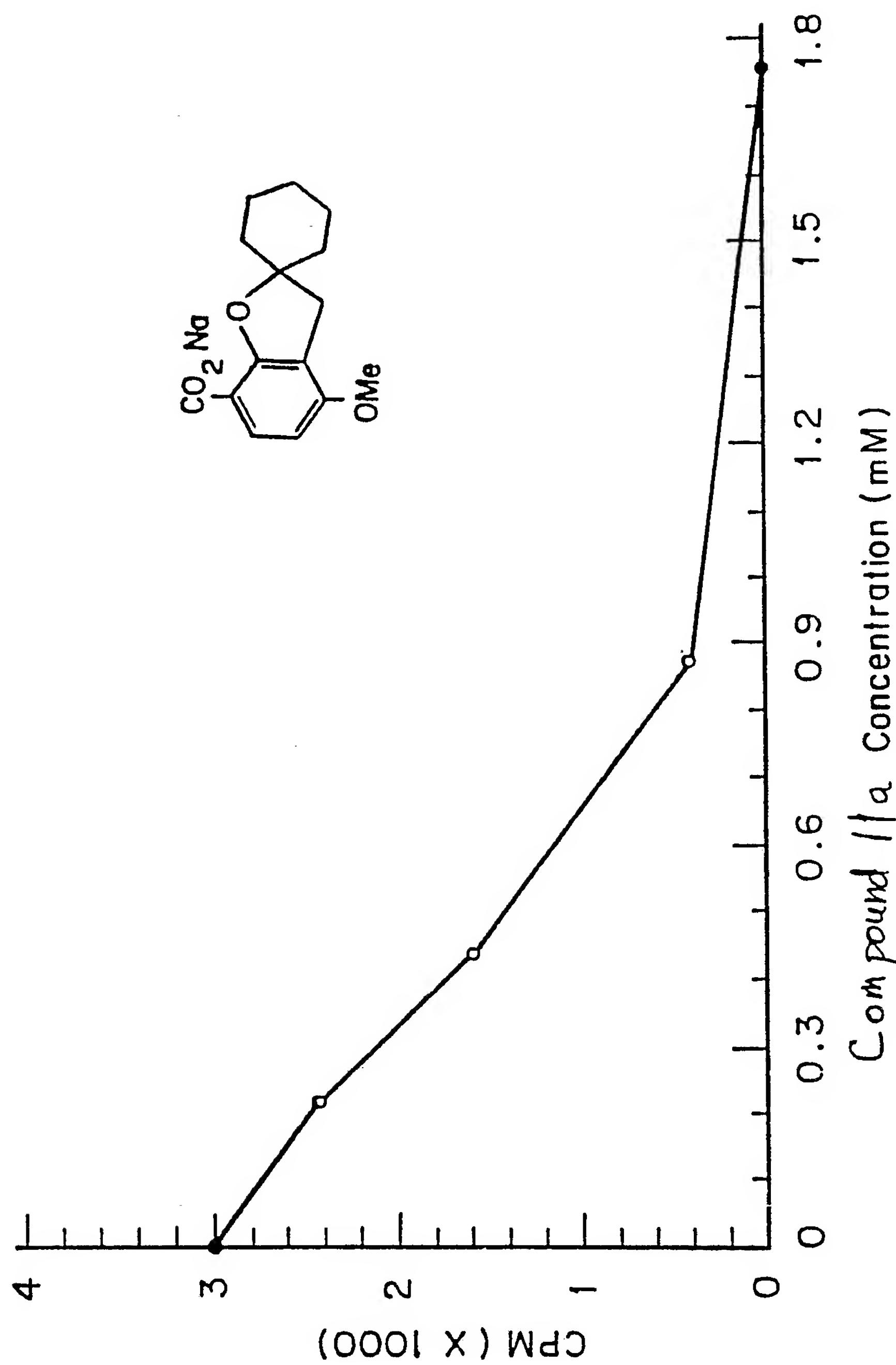
Thymidine Uptake of
PHA Stimulated PBL



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Figure 5

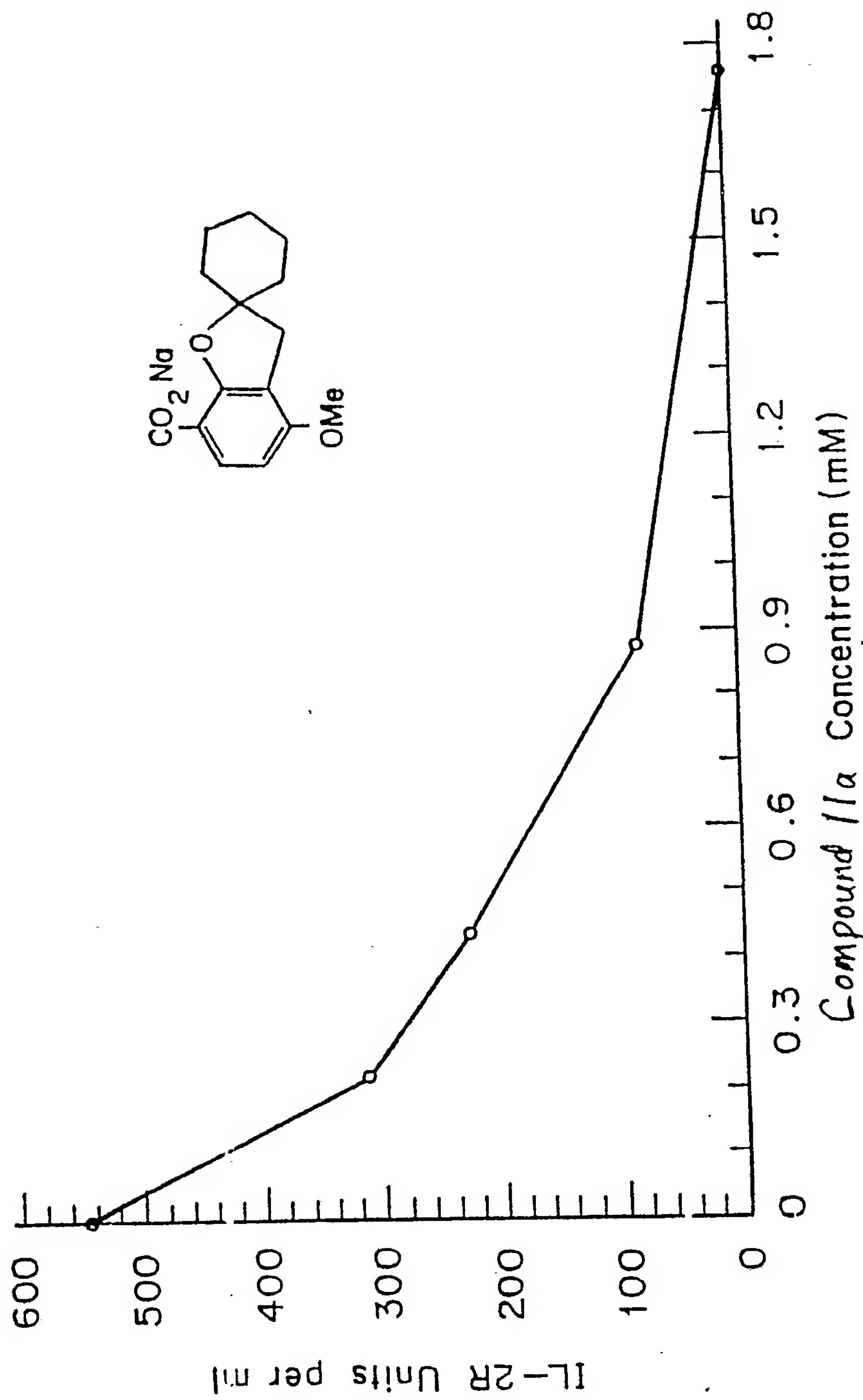
Thymidine Uptake of Anti-CD3
Stimulated PBL



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Figure 6

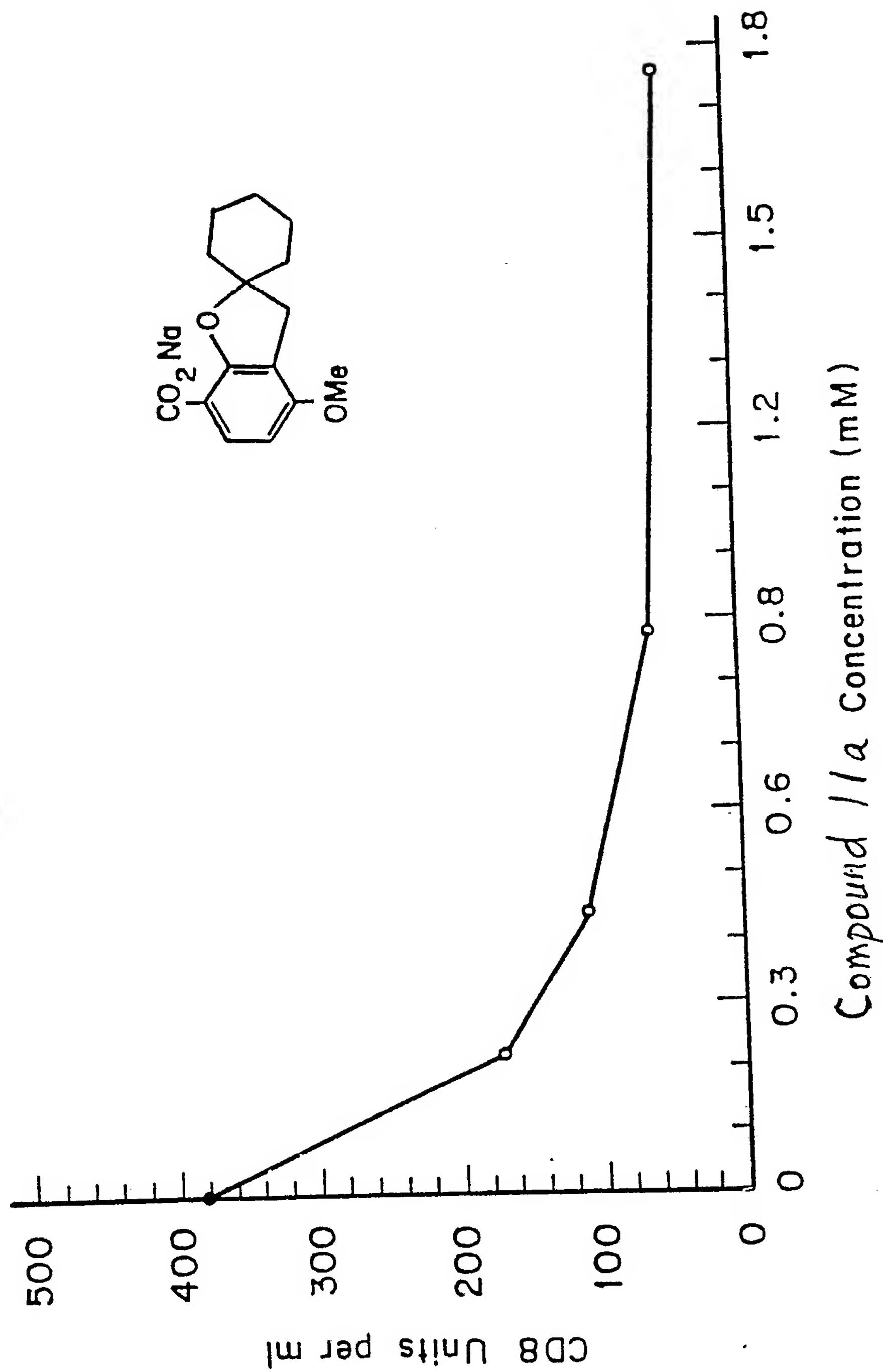
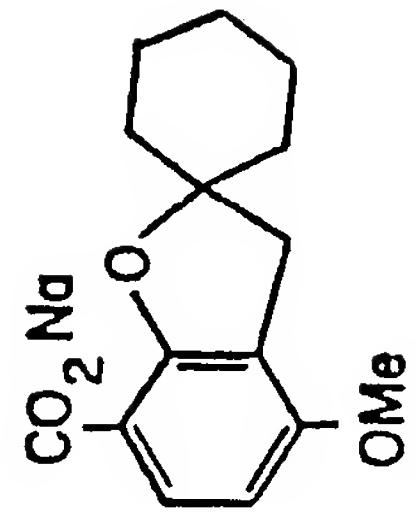
Levels of IL-2R Released From
Anti-CD3 Stimulated PBL



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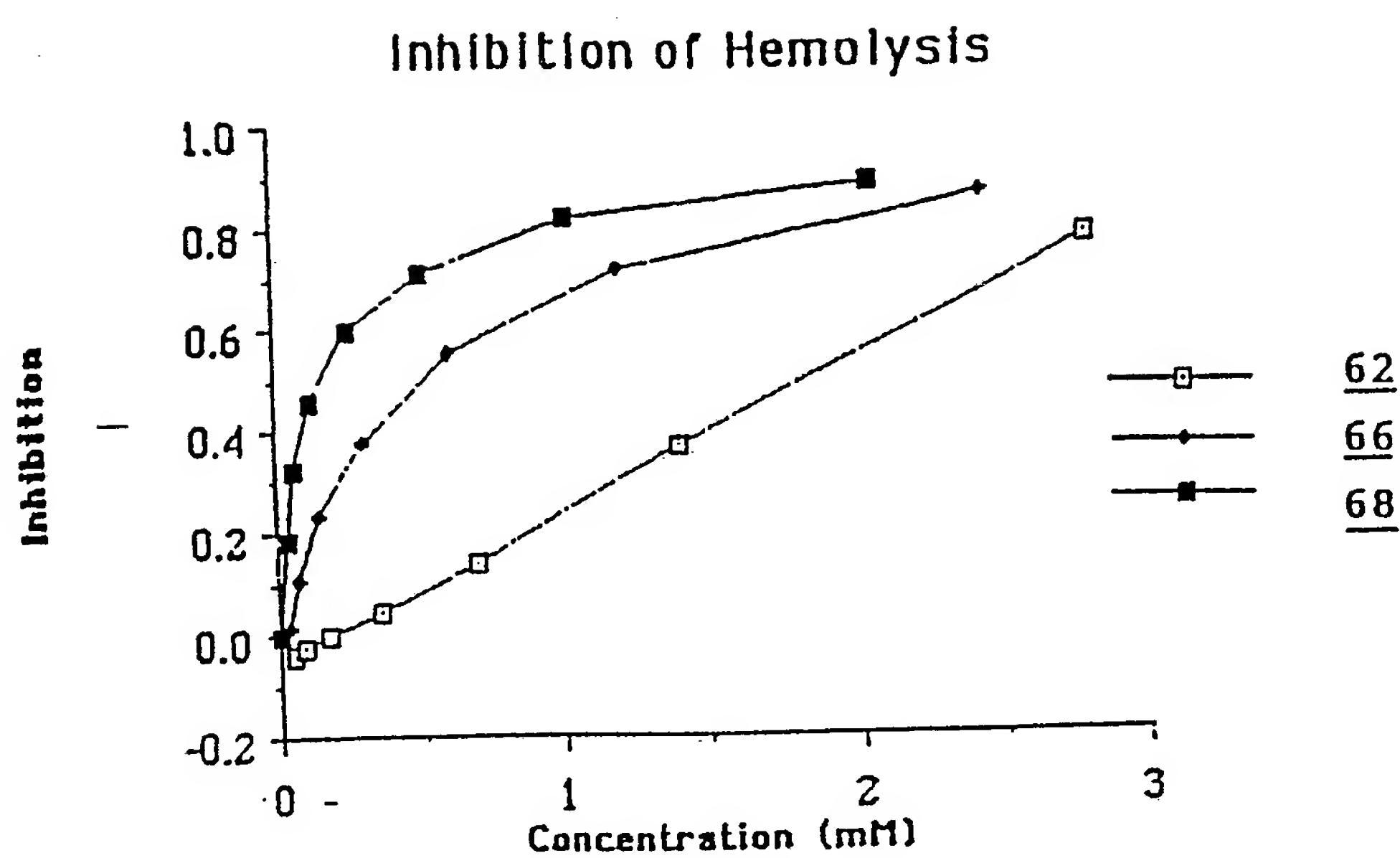
Figure 7

Levels of CD8 Protein Released
From Anti-CD3 Stimulated PBL



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Figure 8



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09303

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC (5): A01N 43/08
 U.S. CL. 514/462

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	514/462, 814, 885, 891

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	US, A, 4,035,509 (NELSON ET AL) 12 JULY 1977 See entire document.	1-16
A	US, A 4,268,516 (LOMBARDINO ET AL) 19 MAY 1981 See entire document.	1-16
A	US, A, 4,740,519 (SHROOT ET AL) 26 APRIL 1988 See entire document.	1-16

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

09 March 1992

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

March 25, 1992

Signature of Authorized Officer

Thurman K. Page